EXPLOITING A NEW ELECTROCHEMICAL SENSOR FOR BIOFILM MONITORING AND WATER TREATMENT OPTIMIZATION

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Abstract: Bacterial biofilm development is a serious problem in many fields, and the existing biofilm monitoring sensors often turn out to be inadequate. In this perspective, a new sensor (ALVIM) has been developed, exploiting the natural marine and freshwater biofilms electrochemical activity, proportional to surface covering. The results presented in this work, obtained testing the ALVIM system both in laboratory and in an industrial environment, show that the sensor gives a fast and accurate response to biofilm growth, and that this response can be used to optimize cleaning treatments inside pipelines. Compared to the existing biofilm sensors, the proposed system show significant technological innovations, higher sensitivity and precision.
Keywords: biofilm monitoring; biosensor; MIC prevention; electrochemically active biofilm; cathodic depolarization.

1. Introduction

Serious wide-range technological problems (corrosion, equipment failure, energy loss, reduced performance and resistance to antimicrobial treatments) can be caused by bacterial biofilm development on any artificial apparatus exposed to natural water (fluid flow systems, water distribution lines, sensors, etc.), with subsequent highly negative economic repercussions (Parr and Hanson, 1965; Whitehouse et al., 1991; Borenstein, 1994; Geesey et al., 1994; Gilbert et al., 1997; Flemming and Schaule, 1994; Schulz and Swain, 2000).

In the water lines of industrial plants, for example, large amounts of disinfectants and other chemical substances are usually employed as a countermeasure against biofilm (Wirtanen et al., 2001; Prince et al., 2002; Maxwell, 2005).

Real-time, continuous monitoring of bacterial growth is extremely useful in order to optimize these (and others) biofilm hindering treatments, making possible to apply them as soon as biofilm appears; regarding chemical treatments, biofilm monitoring allows also the optimization of biocides dosage, entailing a reduction of both costs and of biocide treatments environmental impact.

This led, in the past years, to the study of different biofilm sensing techniques: measurement of *(a)* light scattering (Flemming et al., 1998), *(b)* turbidity (Klahre et al. 1998), *(c)* electrochemical impedance (Muñoz-Berbel et al., 2006; Dheilly et al., 2008), *(d)* vibration response of the monitored surface (Pereira et al., 2008), *(e)* diffusion limitation (Foret et al., 2010). These techniques are affected by several limitations, since all of them:

- can not discriminate between biological and inorganic fouling; this is a major problem, since these two different kinds of fouling require different treatments;
- have a low sensitivity, e.g. can not detect biofilm initial colonization phases, but only thicker bacterial layers; on the other hand, many biofilm related problems, such as Microbiologically Influenced Corrosion (MIC), start as soon as the first bacterial spots appears on a surface (Mollica and Trevis, 1976; Dexter and LaFontaine, 1998; Kimio et al., 2002).

Moreover, some of the above mentioned studies stopped at the laboratory testing phase, and have never been implemented within a real sensor (*c*).

With the aim of overcoming the limitations presented by the existing sensors, a new device ("ALVIM") has been developed (see Section 2.1), exploiting the

cathodic depolarization induced by biofilm growth on active-passive alloys exposed to natural aerated waters. This phenomenon 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. The cathodic depolarization induced by biofilm growth has been largely studied in the last 20 years and has been observed in different parts of the world, both in seawater and in freshwater (Mollica and Trevis, 1976; Scotto et al., 1985; Dexter and Zhang, 1990; Mattila et al., 1997; Dexter and LaFontaine, 1998; Kimio et al., 2002; Wang et al., 2004; Acuña et al., 2006; Dulon et al., 2007; Little et al., 2008). Recently, the electrochemical activity of natural aquatic biofilms was proven to be proportional to the surface area covered by bacteria (Faimali et al., 2008; Faimali et al., 2010), therefore measuring the biofilm electrochemical signal (BES, expressed as current density or potential, see Section 2.1) is possible to know, on-line and in real-time, which is the biofilm covering on a surface.

The aim of this work was to evaluate the performances of this new biosensor, meant to be used for biofilm monitoring and anti-microfouling treatments optimization in industrial environments. The work followed three subsequent steps:

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

(2) Verify the sensor response to biofilm growth in a pilot reverse-osmosis desalination plant, during ordinary working.

(3) Optimization of pipeline chemical cleaning treatments, inside the above mentioned plant, basing on biofilm growth real-time data collected by the sensor.

Biofilm, indeed, represents a major problem in this kind of industrial environment, both for microfiltration (MF) modules and for reverse-osmosis (RO) membranes, increasing management costs (chemical treatments, membranes cleaning) and contributing to cause cloggings which can bring to plant stop (Fritzmann et al., 2007; Vrouwenvelder et al., 2008).

2. Materials and methods

2.1 ALVIM working principle

As described in detail in recent papers (Mollica et al., 1997; Faimali et al., 2008, 2010), cathodic current density $i\ (E,t)$, measured at a given time t on a stainless steel (SS) sample exposed to natural seawater and polarized at a fixed potential E, can be described by the relation:

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

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

Figure 1A shows, schematically, the evolution of the overall cathodic curve (i versus E) during the gradual development of biofilm on the SS surface: curve 1 describes the oxygen reduction kinetics, $i_1(E)$, measured at the beginning of the exposure to aerated seawater on a clean SS surface, whereas

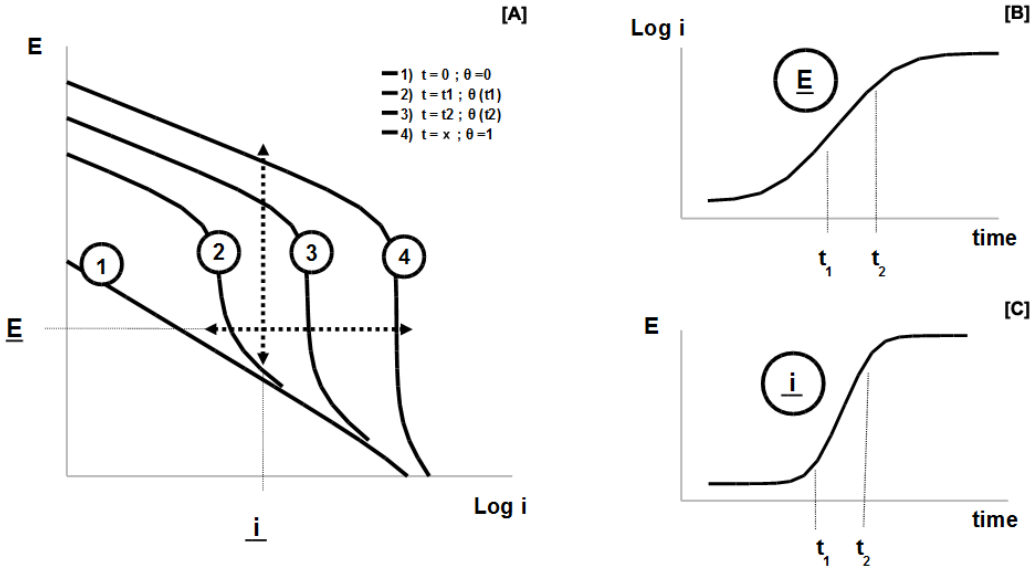

Fig. 1 *– Evolution of the overall cathodic curve (current i versus potential E) during the gradual development of biofilm on a stainless steel surface [A], cathodic currents measured at a fixed potential E [B] and potentials measured at a fixed cathodic current i [C] during biofilm growth.*

curve 4 shows the cathodic curve measured on an SS sample completely covered by biofilm. Curve 2 and 3 describe the trend of the cathodic current in two intermediate conditions.

If, as suggested by 1), the evolution of cathodic current is only due to biofilm evolution, any technique able to signal the gradual cathodic depolarization from curve 1 to curve 4, in Figure 1, can be utilized to build sensors which can provide information on biofilm growth.

At least two classical techniques can be applied to this purpose: a potentiostatic technique or an intensiostatic one.

Following eq. 1, the potentiostatic technique provides information on biofilm development through the measurement of the cathodic currents on an SS sample polarized at a fixed potential *E* (Fig. 1B), whereas the intensiostatic technique provides similar information through the measurement of the potentials able to sustain a fixed cathodic current *i* during biofilm growth (Fig. 1C).

The choice of the most suitable technique between the potentiostatic and the intensiostatic one, in a particular condition or environment, can depend on the specific biofilm-related problem that has to be studied. Potentiostatic polarization was already proved to provide detailed information on the rate of biofilm development (Mollica et al., 1997; Faimali et al., 2008, 2010) from a Θ value less than 1% up to a complete covering of the SS surface.

A possible defect of a sensor based on the potentiostatic technique is that a gradual carbonate precipitation is possible if the high cathodic current requested when biofilm is completely developed (in the order of 50 µA cm^{-2}) is sustained for a long time; it causes, in turn, a gradual decrease of the "active" surface of the sensor which must, hence, be periodically restored by acid cleaning.

The intensiostatic technique, which can operate at cathodic currents lower than 1 µA cm^{-2}, avoids this inconvenient, but provides only the information that a specific biofilm covering threshold (e.g. 10% of the sensitive surface) was reached.

Figure 1C shows, in fact, that the shape of the curve potential versus time is similar to a sigmoidal curve which rises rapidly in a relatively narrow time range [$t_1 < t < t_2$; $\Theta(t_1) < \Theta < \Theta(t_2)$], depending on the selected cathodic current density *i*. The inflection point of the curve can be used to define the threshold value of biofilm covering signaled by the sigmoidal curve obtained at a given cathodic current.

2.2 The biosensor

Basing on the illustrated working principle, the ALVIM sensor can work both in potentiostatic and intensiostatic mode, but, given the previously mentioned considerations, the tests presented here have been performed using the intensiostatic mode. This is considered to be the best one for general industrial applications, since in this working mode the sensor requires less maintenance and gives a clear signal when biofilm covering exceeds the given threshold. In this case biofilm threshold was set to % of the working electrode, to test the device maximum sensitivity.

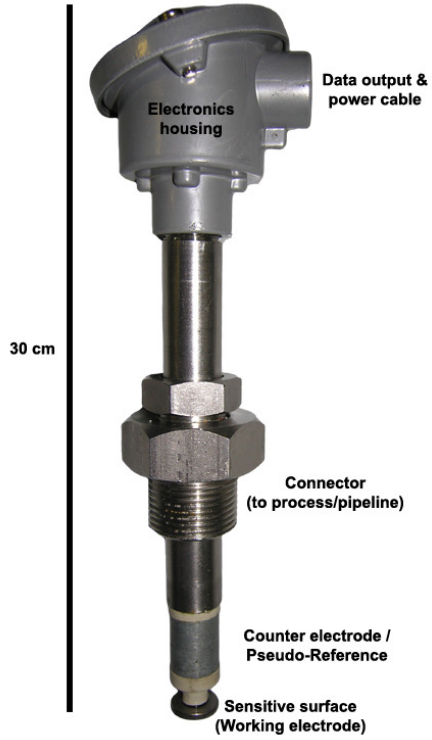

Fig. 2 *– ALVIM biosensor.*

The ALVIM probe (Fig. 2) is compact, requires little periodic maintenance, and can be adapted to fit different kinds of pipeline plug. The sensor is a three-electrodes system, in which the zinc counter-electrode (CE) plays also the role of pseudo-reference (RE). Connected to the zinc and to the stainless steel working electrode (WE), on which 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, the third for data transmission, over local/GSM/GPRS network. This system can be easily scaled and can manage up to several hundreds sensors at a time. The Biofilm Electrochemical Signal (BES, expressed as current

density or potential), measured in real-time, at chosen time intervals, is sent to a remote database. It is possible to graphically visualize data and to raise different alarms in case of BES abrupt changes or achievement of a preset threshold value, corresponding to a defined biofilm covering percentage. The flexibility of this system allows to change electrodes dimensions and shapes, to fit different needs.

2.3 Biosensor preliminary characterization

Laboratory characterization has been performed at the ISMAR Marine Station (member of the European Network of Marine Research Institutes and Stations – MARS), located in the Genoa harbor (Italy), in a tank of about 100 L in which seawater was constantly renewed (1.5÷2 L min^{-1}) with natural water pumped directly from the sea. Three biofilm probe replications have been immersed for about 35 days, during the period September-October (1st year), when seawater temperature ranged from 22 to 24.5°C, and BES was automatically registered every hour. After the reaching of the chosen biofilm covering threshold value (1%), sensitive surfaces were detached from the probe to verify the effective biofilm covering.

2.4 Biofilm covering evaluation

WE surface area covered by bacteria was quantified by means of epifluorescence microscopy and software analysis. After the detachment from the ALVIM probes, each sensitive surface was gently rinsed in seawater sterilized by filtration (Millipore, 0.22 µm pore size), in order to remove unattached cells, then fixed with 2% paraformaldehyde solution for 30min, 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) (Takata and Hirano, 1990), 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 each sample. Images were converted to tiff format (RGB colour) and the surface fraction covered by bacteria was measured, on the 30 images, by means of "Image J" software (Rasband, 1997); mean ± standard error (SE) was then calculated.

2.5 Biofilm monitoring system in-plant testing

The following experiments took place in a FISIA – Italimpianti pilot reverse-osmosis (RO) desalination plant, located at the ISMAR Marine Station too. The pilot plant drew feeding water (1.7 m^3/h) directly from the sea, with a first 100 µm prefiltration and a second 0.1 µm microfiltration (MF). After the MF there was a water storage tank, and then the RO.

Three ALVIM probes were installed, by means of threaded locks, in the plant pipelines (Fig. 3):

- the first in a newly installed pipeline, between prefiltration and microfiltration, where the biofilm was expected to grow sooner;
- the second between MF and the storage tank, where the biofilm was expected to grow later or never, since all the particles larger than 0.1 µm were filtered and this section was treated every few days with strong cleaning agents (NaClO, NaOH and HCl);
- the third between the tank and the RO, where the biofilm was expected to grow, after an initial incubation, because the tank, positioned just before this section, represented a possible large-surface bacteria incubator, more suitable than pipeline for bacterial growth, since water flux was slower and temperature could slightly increase.

These monitoring positions were therefore chosen to obtain a complete view of plant conditions, with reference to biofilm possible problems.

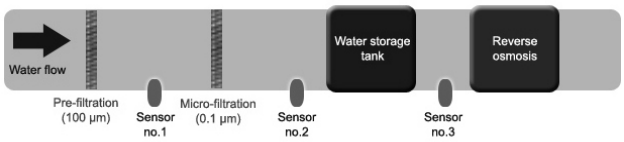

Fig. 3 *– Scheme of FISIA – Italimpianti reverse-osmosis desalination plant and ALVIM probes disposition.*

A first 12-days trial (December of the 1st year) was conducted using all the three above mentioned probes. After some months (July of the 2nd year), a second 12-days trial was performed, employing only the first two probes, in the same plant, to better characterize the differences between biofilm growth dynamics before and after MF, verifying, at the same time, the effectiveness of this filtration against biofilm development. During the last days of this period, continuous chlorination (1 ppm) at the water intake was applied.

In the course of the testing periods, pipelines pressure ranged from 0.2 to 0.8 bar, and seawater temperature from 11.6 to 16°C during the first trial, and from 23.5 to 27°C during the second trial. As for preliminary characterization, after the reaching of the chosen biofilm covering threshold value (1%), probe sensitive surfaces were detached from the probes to

verify the effective biofilm covering.

2.6 ALVIM system as a chlorination triggering device

The subsequent step, a few months later (December of the 2nd year), was the optimization of pipelines chemical cleaning treatments inside the above mentioned pilot plant, basing on biofilm growth real-time data collected by the ALVIM system. For this aim, biofilm growth signal from sensor no.1 was employed as a trigger to remotely start the 1ppm chlorination at the water intake. During the 20-days testing period, pipelines pressure ranged from 0.2 to 0.8 bar, and seawater temperature from 11 to 15°C.

3. Results and discussion

3.1 Biosensor preliminary characterization

During preliminary testing, after just a few days of immersion in the seawater tank, biofilm probes showed an increase of the BES from around 500 mV *Vs.* Zn to more than 1100 mV *Vs.* Zn (Fig. 4), corresponding to a biofilm covering, quantified by microscopic analysis, of 3-4% of the WE surface. Considering that the sampling has been done some days after the reaching of the threshold value (marked in Fig. 4 by an asterisk), these data fit well with the chosen biofilm covering threshold (1% of the WE surface). These results are consistent both in terms of BES evolution curves (reasonably low standard error among replications and few differences among subsequent repetitions) and of biofilm covering (actual data match expected values), confirming that the ALVIM sensor worked reliably over the considered time period.

3.2 Biofilm monitoring system in-plant testing

After preliminary experiments, ALVIM testing proceeded in the pilot reverse-osmosis desalination plant (Fig. 5). After 2-5 days, BES of biosensors no.1 and no.3 started to increase, signaling that the biofilm covered more than 1% of WE surface. The BES rise occurred later in the stretch crossed by just prefiltered water (sensor no.1), but with a new and clean pipeline, than in the section after MF, between tank and RO (sensor no.3), never cleaned. This highlights the fact, suggested also by other experimental evidences (Donlan, 2002; Nikolaev et al., 2007; Sriyutha Murthy and Venkatesan, 2009), that already existing biofilm, inside pipelines, can have more influence in biofilm propagation/development than new bacteria transported by feeding water, underlining the importance of an appropriate pipeline periodic cleaning. Sensor no.2, positioned in a section treated every few days with strong cleaning agents, did not show any biofilm growth signal, indeed. Microscopic examination showed that, after nine days of immersion, biofilm covering percentages on sensitive surfaces no.1 and no.3 were, respectively, about 1% and 2% (matching the chosen threshold of 1%). After the sampling, the plant was stopped, the section between MF and water storage tank was chemically cleaned (NaClO, NaOH); sensitive surface no.3 was replaced, while sensor no.1 was disconnected, for technical reasons related to the plant. At the end of the 12-days testing period, sensitive surfaces no.2 and no.3 (the last one replaced after the sampling on day 9) were nearly clean. The lower biofilm covering

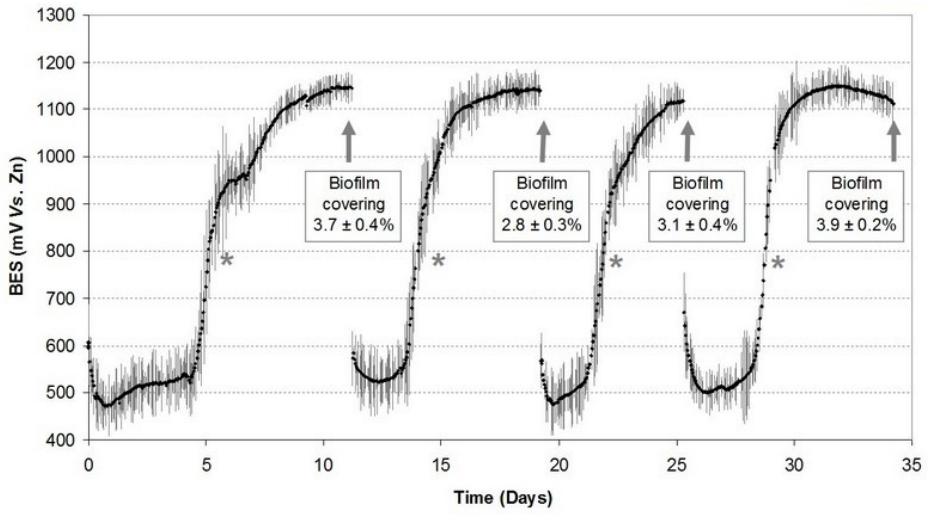

Fig. 4 *– ALVIM probes BES evolution (mean ± SE, mV Vs. Zn) during preliminary testing in a tank with constantly renewed natural seawater. The asterisks mark the reaching of the chosen biofilm covering threshold (1% of the WE), the arrows mark sensitive surface sampling/analysis (biofilm covering data are indicated in the boxes, as % ± SE, calculated on the three replications) and replacement.*

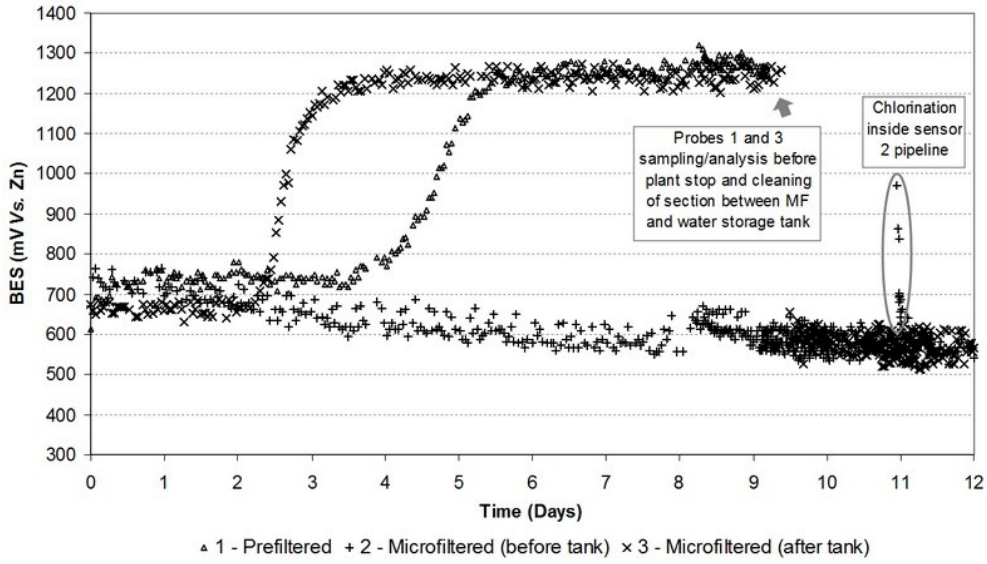

Fig. 5 *– ALVIM probes BES evolution (mV Vs. Zn) during the first test in a pilot reverse-osmosis desalination plant. The arrow marks sensitive surfaces no.1 and no.3 sampling/analysis before plant stop and cleaning of the section between MF and water storage tank; on sensor no.3 a new sensitive surface was installed. The oval marks a chlorination in the same pipeline.*

of sensitive surfaces no.1 and no.3 sampled on day 9, compared to those observed during preliminary testing (see Section 3.1), could be due to the different conditions, such as temperature, light, water filtration. The quick and precise ALVIM system response to biofilm growth, observed in the course of the preliminary characterization, was therefore confirmed during in-plant testing.

Moreover, the BES showed a clear peak in correspondence to chlorination (marked in Fig. 5 by an oval), suggesting that ALVIM could also be used to monitor chlorine-based treatments.

During the second testing period (Fig. 6), the signal of sensor no.1 started to increase one day earlier than in the first testing and, thereafter, grew at an higher rate. This reflects the different seasons, the different natural biological activity and, likely, possible differences of nutrients load in the water during the two tests, since the first one took place in December, while the second one in July. This observation

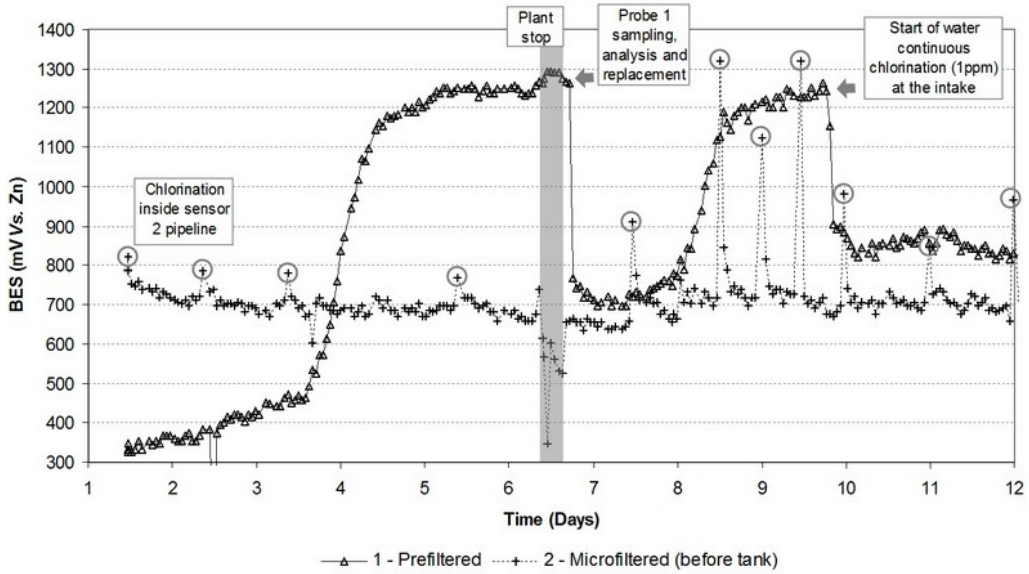

Fig. 6 *– ALVIM probes BES evolution (mV Vs. Zn) during the second test in a pilot reverse-osmosis desalination plant. The circles mark chlorinations in the section between MF and water storage tank. The gray area marks plant stop for maintenance. The arrow on day 6 marks sensitive surface no.1 sampling, analysis and replacement. The arrow on day 9 marks the start of continuous chlorination (1 ppm) at the intake (whole pipeline treatment).*

confirms the flexibility of the employed biofilm monitoring system, underlining at the same time the impossibility of adopting a "one-for-all" cleaning treatment approach.

The signal of sensor no.2 did not increase over the baseline, except for the peaks observed when the signal was acquired during or nearby the chlorinations of the pipeline in which the sensor was placed. The height of those peaks was related with the proximity of the data acquisition moment with the chlorination time. The bio-electrochemical signal of sensor no.2 confirmed that the cleaning treatments carried out in this pipeline were effective against the biofilm; the bacterial covering of probe no.2 WE, at the end of the testing period, was nearly 0%, indeed. These observations sustain the effectiveness of ALVIM as monitoring device for chlorination treatments, as suggested by the data of the first testing period.

During plant stop for maintenance (gray area in Fig. 6), the signal of sensor no.2 showed a drop, because the pipeline in which that sensor was placed got empty (no water). Just before plant restart, probe no.1 WE was sampled and replaced. The biofilm covering percentage was about 1% , fitting well, as the previous data, with the chosen threshold value.

On day 9, a chlorination treatment (continuous, at 1 ppm) at the water intake was started, and the signal of sensor no.1, on which the biofilm had grown, nearly immediately decreased, confirming the effectiveness of that treatment . The signal remained 100-150 mV over the baseline, because of the chlorine continuous presence in the water. Chlorine was likely "consumed" before sensor no.2 by the organic matter present inside microfiltration module and pipelines, in fact this sensor did not show any signal increase. As previously mentioned, at the end of the testing period, biofilm covering on probe no.2 WE was nearly 0%.

3.3 ALVIM system as a chlorination triggering device

During the last testing period, in line with what was observed during the above mentioned tests, after two days of incubation the BES started to grow (Fig. 7), signaling that the biofilm surface covering, on the probe WE, reached the chosen threshold (1%). On day 4, the first ALVIM-triggered chlorination was started; the treatment time was set to 30 minutes. Immediately after this chemical cleaning treatment, the BES dropped to the initial value (around 700 mV *Vs.* Zn).

In about two days, the biofilm growth signal increased again, and this time the 30-minutes chlorination was started in advance with respect to the previous one (the BES was around 900 mV *Vs.* Zn, while on day 4 it was nearly 1100 mV *Vs.* Zn). After the treatment, the sensor WE was immediately sampled and replaced. Biofilm covering on the sampled surface, quantified by laboratory analysis, was about 1%, matching the expected value. This evidence highlights the fact that, obviously, the applied chlorination treatment did not imply an immediate detachment of the biofilm from the surface.

After WE substitution, it took four days to the BES to start growing again. Chlorination time was extended

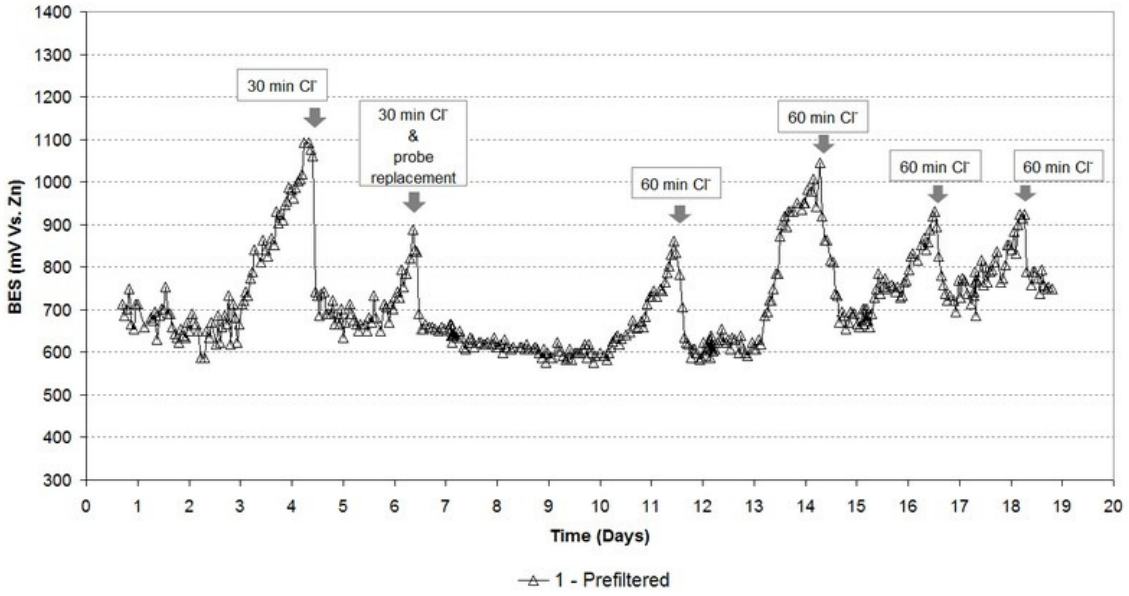

Fig. 7 *– ALVIM probe BES evolution (mV Vs. Zn) during the use of ALVIM system as a chlorination triggering device. The arrows mark pipeline chemical cleaning by means of water chlorination (1 ppm) at the intake.*

to 60 minutes, to verify the requested treatment frequency with a longer treatment time. Chlorination frequency continued to be about one treatment every two days, and, moreover, after the chemical cleaning performed on day 14 the BES did not return to the initial value, showing an increased after-treatment bio-electrochemical activity. This meant that the biofilm was not completely inactivated/killed by the chlorination, and implied also the need of more frequent treatments. Such information is essential to adjust timing and dosage of chemical cleaning, since even the survival of a small part of the settled bacteria is a guarantee of the fact that the biofilm will quickly grow again, thanks to the replication of the microorganisms still alive.

These data suggest that, compatibly with environmental impact consideration and by law limit, higher chlorine concentrations or longer treatments had to be used, in this case, to completely remove the biofilm from the internal surfaces of plant pipelines.

At the same time, the ALVIM system demonstrated its usefulness for the monitoring of biofilm growth and consequent cleaning treatments optimization inside water pipelines.

3.4 ALVIM compared to the other biofilm monitoring devices

Comparing ALVIM sensor to the other biofilm monitoring devices, such as those, discussed in the introduction, based on light scattering (Flemming et al., 1998), turbidity (Klahre et al. 1998), electrochemical impedance (Muñoz-Berbel et al., 2006; Dheilly et al., 2008), vibration response of the monitored surface (Pereira et al., 2008) and diffusion limitation (Foret et al., 2010), it possible to see that the experimental results of this new device testing highlight significant advantages:

- the possibility of monitoring just the biological fouling (microfouling), discerning it from the inorganic fouling; the above mentioned sensors, indeed, are not able to discriminate between these two different kinds of fouling;
- the detection of the biofilm since its first colonization phase (i.e. the first bacterial layer); the above mentioned sensors detect only thicker (several µm) biofilms.

Among the sensors based on electrochemical techniques, the most known are BIoGEORGE (Licina and Nekoska, 1993) and BIOX, born, earlier than ALVIM, from the same research activity (Cristiani et al., 1998). They usually show an higher sensitivity, with respect to those based on the previously mentioned techniques, but no clear quantitative data has been found in literature.

Considering device flexibility, both BIoGEORGE and BIOX have a fixed working mode and sensitivity, while ALVIM can be set to monitor different extents of biofilm covering and can work both in intensiostatic and in potentiostatic mode. The last one, discussed only marginally in this work, will be the subject of future studies.

From the technical point of view, the electronics of BIoGEORGE for the control, data acquisition and data analyses are housed in an external box, where the readings are stored in a database (DB). In this way data are not available in real-time from remote, but has to be downloaded to a PC. The BIOX sensor needs external hardware too, moreover device control and data reading are basically analogical (Cristiani et al., 1998, 2000; Cristiani, 2005). On the other hand, ALVIM has a fully digital management, and its electronic is completely integrated within sensor housing; in industrial environments, indeed, device compactness represents a valuable advantage. About data storage, ALVIM uses a remote DB, thus the collected information can be viewed in real-time even from remote.

4. Conclusions

Experimental results show that the ALVIM system works reliably in a real industrial environment, representing an efficient biofilm monitoring solution; it gives a fast and accurate information about the bacterial covering, even at early stages of colonization.

Furthermore, the data provided by this system proved to be very useful if applied to cleaning treatments optimization, enabling to hinder biofilm growth as soon as it starts.

This is a promising technology in any field affected by biofilm-related problems, prefiguring a wide application range for the ALVIM system.

Next biosensor developments will concern longer trials, experiments in different conditions (e.g. freshwater, other industrial environments) and testing of new materials for biosensor components.

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