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A new Model System for Monitoring the Biofilm Growth and its Application in Industrial Processes

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Biofilm is defined as a complex multi-species microbial community highly and specifically organized attached to the surface through an hydrated polymeric matrix known as EPS (extracellular polymeric substances), which represents the primary constituent of the biofilm. The industrial problems associated with the biofilm growth are the subject of many studies concerning the water system and the cooling tower. Cooling towers are very important part of many industrial systems, like refinery and gas extraction plant. The driving force for heat transfer is the temperature difference between the two components in direct contact or separated by surfaces, which are at different temperatures, and the decrease of this parameter can negatively affect the entire process. The biofilm formation on heat exchanger surfaces of the cooling towers, known as biofouling, strongly reduces the heat transfer efficiency. The monitoring of the benthonic communities growth within a cooling tower is not an easy task due to logistical issues mainly related to sampling difficulties. This study aims to realize a biofouling control strategy that allows a more efficient biomonitoring of the biofilm growing in a cooling tower. The developed monitoring system was tested using samples of make-up water taken from a cooling tower at a full scale Refinery located in Northern Italy. This experimental apparatus (Model System) allowed the monitoring of biofilms growth that develops from make-up water entering cooling tower and to test the effectiveness of several additives that are usually used in real systems, in order to control the development of biofouling. The refinery make-up water was characterised in order to describe the composition and the structure of the microbial communities by applying in situ hybridization techniques (Fluorescence in Situ Hybridization, FISH). The refinery make-up water fed a continuous recirculating system [maximum volumetric flow rate=2 mL/min] that exploits a "Coupon evaluation Flow Cell (BST FC 71©Biosurface Technologies Corporation)" made in black anodized aluminium, with a single flow channel, that uses standards microscope coverslips as a viewing window. Through this window, it is possible to monitor over time the growth of the biofilm on the coverslips surface placed inside the flow chamber by a simple phase contract microscopy analysis (1000X). The biofilm formation rate was estimated during the early phase of biofilm formation ($r_{bf\ (5\ days\ growth)}$ =1.4*10^4 cells/mm^2*d) and after a prolonged period ($r_{bf\ (20\ days\ growth)}$ =2.4*10^4 cells/mm^2*d).The effectiveness of additives provided by Chimec S.p.A. was also tested in order to evaluate the impact on biofilm formation.

1. Background and aims

1.1 Biofilm formation

Biofilm formation is a topic widely studied in several fields ranging from infrastructures, such as plumbing, oil refineries, paper mills, heat exchangers to medical implants and the annual cost due to its containment is approximately on the order of billions of euros.
Biofilms are complex communities constituted by microorganisms adhering to surfaces trough the production of a mucilaginous matrix highly hydrated and mainly composed by exopolymers. The exopolymeric matrix (approximately 90% of biofilm dry weight) is composed of 70% water and the remainder of polysaccharides, proteins, nucleic acids and lipids, which overall are called EPS, Extracellular Polymeric Substances.
Biofilm may be composed either of a limited number of microbial species or very large variety of organisms such as bacteria, fungi, algae, yeasts and sometimes even small protozoa and metazoa. The microorganisms that compose the biofilm may have a phenotypic and genetic diversity which ensures them an evolutionary advantage and an ecological advantage compared to suspended communities (Battin et al. 2003). Notwithstanding the great biodiversity of these benthonic communities, the biofilm

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formation can be described in five stages. The initial stage of attachment involves the adhesion of pioneers' bacteria, through electrostatic attraction and physical forces. The first colonizers facilitate the arrival of other cells, providing multiple sites of cell adhesion (second stage of irreversible attachment) and starting to produce a polysaccharide matrix, which helps the maturation of the biofilm (third and fourth stage of maturation). The biofilm grows and incorporates bacteria and other external organisms until the last stage which involves the breaking of the protective matrix and the dispersion of the cells. The lysis of the polysaccharide matrix can also be caused by external agents, such as biocides or biodispersants, but if the removal of biofilm is not complete, the growth and maturation of new biofilm occurs more quickly (Flemming 1991), and for this reason, to get a complete removal, it is often necessary mechanical action.

1.2 Biofouling in cooling water system

In a cooling system, all the system components are exposed to the process water containing benthonic organisms. This complex phenomenon, known as biofouling, is caused by the growth of bacteria, fungi, algae, and higher organisms which, under optimal growth conditions, develop by remaining immobilized to the surfaces of the pipes of industrial plants, inside the heat exchangers and the internal surfaces and fills of cooling towers (Lutterbach et al 1996).

Biofilm allows the adhesion of higher organisms and may cause several problems in cooling water. The metal surfaces are attacked by the microbiologically induced corrosion (MIC) (Videla and Characklis 1992), the resistance to heat transfer within the heat exchangers is increased because the thermal conductivity of the biofilm is significantly lower than the metal one; the increase of surface thickness increases the frictional resistance of the fluid; environmental conditions within these systems (hot and humid) promote the development of pathogenic organisms, such as *Legionella pneumophila*.

These problems involve a significant loss of energy and a considerable economic impact. As described by Murthy and Venkatesan in 2008, the biofilm growing within industrial systems may greatly differ because it may be influenced by several factors like working temperature (Bott 1995), availability of nutrients (Griebe and Flemming 1998, Flemming 2002), flow rate (Adamczyk 1981), substrate of adhesion and suspended solids (Bott and Melo 1992). Biofouling control in industrial systems is a key point of industrial water treatment. Usually biofouling is thwarted by using oxidizing and non-oxidizing biocides. The main biocide products used industrially for controlling the phenomenon of biofouling are oxiding like bromine, chlorine, chlorine dioxide and ozone, or non-oxiding like alkyl dimethyl benzyl ammonium chloride (ADBAC), β-bromo-β-nitrostyrene, 2-bromo-2-nitropropano-1,3-diol (BNPD), Chlorophenols. Large amount of these products are directly added into the flow of the cooling system water (Murthy et al. 2008).

These additives are toxic to the environment and their dosages should be constantly and closely monitored.

1.3 Monitoring of Biofouling in cooling water system

Effective management of cooling water involves the control of biofilm growth through the application of a correct dosage of biocides and the periodic surface cleaning.

The main methods used to estimate the components of microbial communities involved in biofouling are shown in Table 1.

Table 1. Methods utilized for analyzing microbial components of biofilms (Murthy and Venkatesan 2008).

Biofilm Parameters	Method	References
Direct cell counting	Epifluorescence microscopy	Daley and Hobbie (1975)
Biofilm thickness	Light microscopy	Blakke and Olson (1986)
Colony forming units	Standard method	APHA (1995)
Total living biomass	Adenosine triphosphate Fluorescein diacetate estimation	Chalut et al. (1995) Rosa et al. (1998)
Total biomass	Total organic carbon	
Dry weight	Biofilm total suspended solids	APHA (1995)
Algal biomass	Chlorophyll and phaeophytin estimation	APHA (1995)
Total proteins	Protein determination	Bradford (1976)
Total sugars	Carbohydrate determination	Dubois et al. (1956)
Lipids	GC-MS	Geesey and White (1990)
Uronic acids	Uronic acid determination	Mojica et al. (2007)
Respiratory activity	CTC staining method	Schaule et al. (1993)

The interior of the cooling towers is unlikely to be sampled and inspected and therefore the growth of dispersed microbial populations in the recirculating cooling water is routinely monitored. The

microbiological characterization of these systems is commonly performed by means of ready-to use kits which are easily interpretable and provide the biomass concentration generally in terms of CFU/mL. The latter are often not rigorous and therefore they do not enable an efficient monitoring of biofilm development.

2. Characterization of make-up water

The water sample used for experimentation comes from the make-up of a cooling tower of a refinery located in Northern Italy. The make-up water was stored at room temperature and protected from direct light. The chemical characterization of make-up water is reported in Table 2.

Table 2. Analytical results of chemical characterization of make-up water used for experimentation.

Parameter	Value
Total Carbon	30.75±0.05 mg/L
Turbidity	10.9 UA
pH	8.7
Electrical conductivity	$3.86*10^3$ μS
VSS	0.033 mg/mL
FSS	0.038 mg/mL
TSS	0.071 mg/mL

FISH analysis (Fluorescence in Situ Hybridization) was also performed on feeding water in order to evaluate the main microbial components. Samples were immediately fixed in formaldehyde and ethanol as described in FISH Protocol (Daims et al. 2001) in order to estimate the bacterial abundances by means of EUB338mix probes specific for Bacteria domain (Amann et al. 1995). The 4',6-diamidino-2-phenylindole (DAPI) fluorescent staining was also performed routinely for determining total cell numbers, from which the relative percentage abundances of the bacterial population was calculated. FISH quantification of hybridized cells was performed by epifluorescence microscopy (Olympus, BX51) by counting fluorescent cells (at least 100 cells per grid) on random grids on glass slides. Images were captured with an Olympus XM10 camera and analysed using Cell-F software (Olympus, Germany). Error bars were calculated as standard deviations of cell counting performed on at least 10 microscopic grid for each filter; all samples were analysed in duplicate. The obtained values are expressed as relative abundance of cells out of the total biomass present in the sample.
Bacteria and total cell estimated on samples taken at the beginning of experimentation (April 2013) and after three months (July 2013) are reported in Table 3. FISH assay showed that more than half of the total biomass was likely active and belongs to Bacteria domain.

Table 3. Analytical results of microbiological characterization of make-up water used for experimentation; the values are shown with standard deviation.

	April 2013	July 2013
Total cells [cells/mL]	$2.04*10^6±4.52*10^5$	$2.59*10^6±1.48*10^5$
Bacteria [cells/mL]	$1.04*10^6±2.95*10^5$	$1.71*10^6±9.61*10^5$
% Bacteria/Total cells	50.9	66.0

3. The Model System Apparatus developed

This research project aims to validate a simple and efficient method of monitoring biofouling in cooling towers. The first phase of the study was focused on the construction of an instrumentation model at laboratory scale that allows, using samples taken from an industrial operating plant, to study the formation and maturation of the biofilm.
By adapting the experimental apparatus previously described (Sjollema in 1988 and 1989; Jackson in 2001) a circulating system was constructed (Figure 1) continuously fed with make-up water as described in the previous paragraph. This system uses a flow cell (BST FC 71 © Biosurface Technologies Corporation) made of anodized aluminum, provided with a single flow channel and a viewing window. Through this window, the biofilm growth can be monitored over time by optical microscopy in contrast phase (1000X) on the surface of a standard microscope cover slips, chosen as adhesion substrate, located inside the flow chamber.

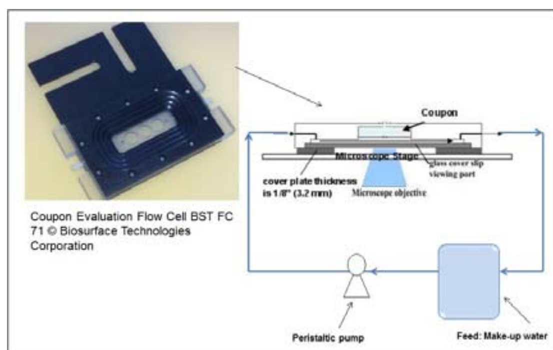

Figure 1: scheme of the Model System Apparatus developed in the experimentation.

4. Results

4.1 Evaluation of biofilm formation

The biofilm development on the inner surface of the cover glass located in the flow chamber was monitored using optical microscopy and estimated in terms of cell abundance increase over time (direct cell counting).
Using make-up water as inoculum, biofilm formation rate [cells/mm^2*d] was evaluated by this formula:

$$r_{bf} = \frac{d(cellular\ abundance)}{dt}$$

Several tests were performed by keeping constant the temperature (T= 20°C), the maximum volumetric flow rate (2 mL/min) and by protecting the system from direct sunlight exposure.
The biofilm formation rate was estimated by evaluating the biofilm growth on different glass coverslips located within the flow chamber. Figure 2 shows microphotographs of the different growth phases of biofilm taken with phase contrast microscopy. At different sampling times (after 2 days, 4 days, 6 days, 15 days and 20 days). The biofilm growth was very fast; only after seven days the direct cell counting becomes difficult to perform. After two weeks, the coverage of coverslips was almost complete. The time trend of cellular abundance was studied placing one, two or three coverslips within the flow chamber (three replicate tests). Biofilm formation rate estimated at 5 days ranged between 1.26*10^4 and 1.49*10^4 cells/mm^2*d. The experiment performed for a longer period (20 days), provided an average value of r_{bf}=2.37*10^4 cells/mm^2*d. When biofilm becomes 'mature' (after 6-9 days) the direct cell counting becomes less smooth; we chose to perform detachment tests with additives, as described in paragraph 4.2, by growing biofilm for 4 days.

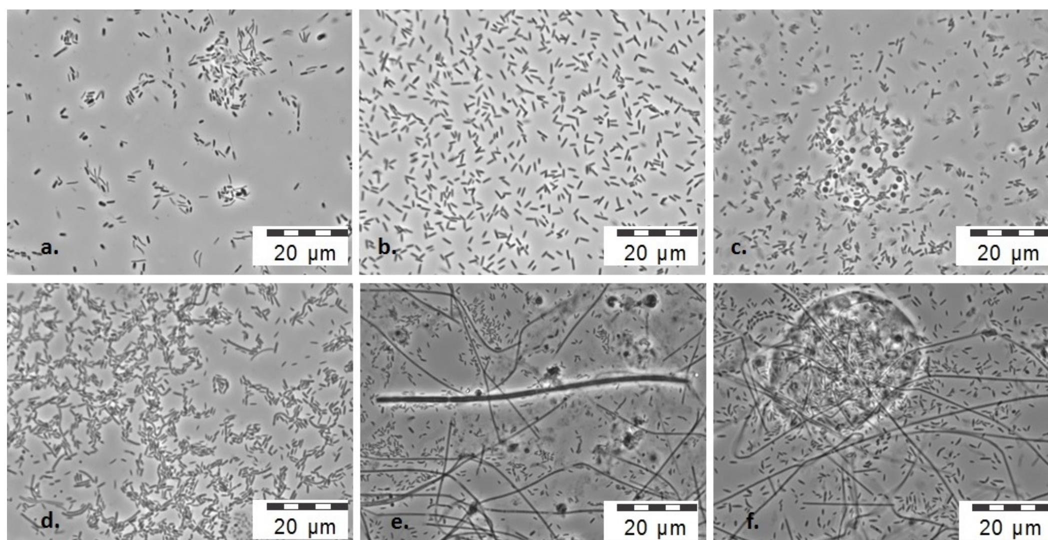

Figure 2. Micrographs taken with phase contrast microscopy during different phases of biofilm growth (a. after 2 days; b. after 4 days; c. after 6 days; d. after 9 days; e. after 15 days; f. after 20 days).

4.2 Detachment tests with additives

The effectiveness of additives provided by Chimec S.p.A. was tested using the developed Model System apparatus: a mixture of anionic and non-ionic surfactants with dispersing properties (Chimec 7464), a biocide with dispersing properties containing Benzalkonium chloride (Additive 2) and a common non-oxidizing biocide without dispersing properties (Glutaraldehyde). The tests were performed on biofilms grown on glass surface after recirculating make up water throughout the system. Several additives at an established concentration were directly added to the recirculation make up water. Tests were performed keeping constant the temperature (T=20°C), the maximum volumetric flow rate (2 mL/min) and protecting the system from direct sunlight exposure. The biofilm detachment from glass surface was monitored on inner surface of three coverslip placed inside the flow chamber by evaluating the surface cell density by direct cell counting at different incubation times (initial time of the test, after 3 and after 5 hours). Table 4 shows the additives concentration chosen to perform tests. The impact of four different additives on either the detachment of mature biofilm or on the biofilm formation rate are reported in Figure 3 a. and b. respectively. The biofilm detachment rate (r_{bd}) is reported for each additive in Table 4. The biofilm detachment rate is expressed as n. cells/mm^2*h and was estimated in triplicate tests for each screened additive. The impact of four different additives on either the detachment of mature biofilm or on the biofilm formation rate are reported in Figure 3 a. and b. respectively.

The tested additives showed comparable and linear biofilm detachment rate, with the exception of the mixture of Chimec 7464 with Additive 2, even though it was able to remove 50% of the biofilm. A lower biofilm detachment was observed when single additives were used (about 30 % for Chimec 7464 and for Additive 2). Only Glutaraldehyde allowed to reach 60% of biofilm removal.

It is worth to noting that the biofilm formation was observed also in the presence of additives as shown in Figures 3b. In the presence of additives, after 20 hours, the cell abundance reached a mean value of 4.6*10^4 cells/mm^2. As shown in Table 4, the most effective additives were Chimec 7464 and the mixture Chimec 7464/additive 2; they indeed more efficiently hindered the biofilm growth. Overall, in the presence of all tested additives, the biofilm formation rate (r_{bf^*}) was an order of magnitude lower than r_{bf} calculated recirculating only make-up water as inoculum. Furthermore, a successive biofilm detachment was also obtained with all additives with a biofilm detachment ranging between 40% and 65%.

Table 4: Summary of the additives used in this study reporting the employed concentrations, the related biofilm detachment rate (r_{bd}) and the related biofilm formation rate (r_{bf^*})..

Additive	Concentration [ppm]	r_{bd} [cells/mm^2*h]	r_{bf^*} [cells/mm^2*h]
Chimec 7464	1000	8.32*10^3	1.3*10^3
Additive 2	500	7.61*10^3	2.7*10^3
Glutaraldehyde	500	8.55*10^3	2.2*10^3
Chimec 7464+Additive 2	1000+500	4.80*10^3	1.4*10^3

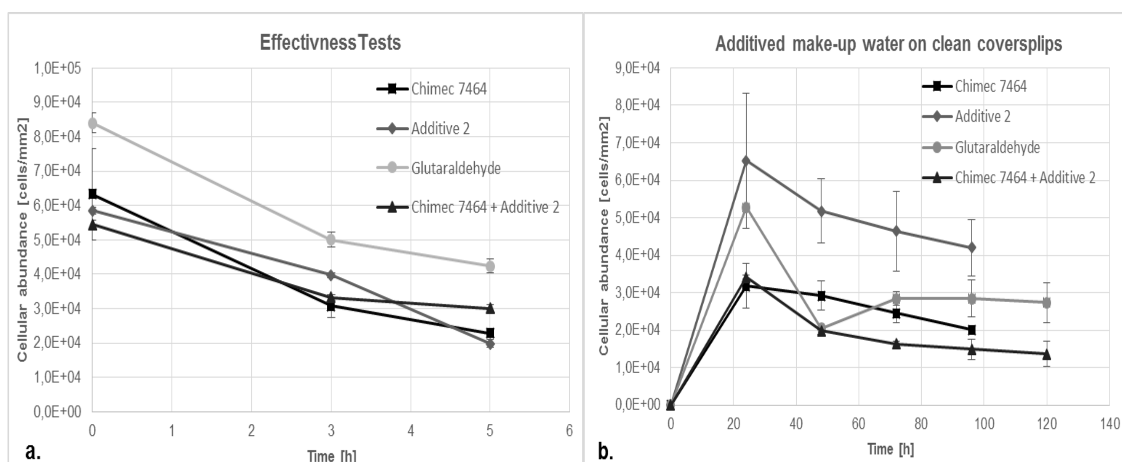

Figure 3: Impact of additives addition on biofilm (a.) and biofilm growth on obtained recirculating make-up water containing additives (b.).

5. Conclusions

The Model System Apparatus allowed to efficiently and easily monitor biofilm growth, using make-up water of a cooling tower located in Northern Italy. The effectiveness of the additives was clearly shown and investigated on either 'mature' biofilm or during the biofilm formation. Overall, the system is versatile and may be employed to study and control biofilm formation under different operative conditions (i.e. additives concentration, residence time, biofilm with higher thickness). Present study showed that the mixture of Chimec 7464 with Additive 2 was more efficient than other studied additives (removal percentage=55%) on biofilm growing on recirculating make-up water as inoculum through the Model System, excluding the glutaraldehyde for its poor environmental compatibility.
Tests performed by recirculating make-up water with additives showed that Chimec 7464 alone or in combination with Additive 2 were most efficient among those tested in this study (removal percentage=65%).

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