Study of biofilm associated bacteria on polyvinyl chloride, stainless steel and glass surfaces in a model cooling tower system with different microbiological methods

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Abstract

Cooling towers are an integral part of large range of industrial processes. The conditions of cooling water systems provide an ideal environment for microbial growth and biofilm formation. Microorganisms which enter via makeup water or air to the system form a biofilm layer on the inside surfaces of cooling tower which contain water and limited nutrients. Biofilm formation in cooling tower systems is undesirable for operational and public health reasons. Thus, microbial load should be monitored and kept under control. In this study, bulk water and biofilm associated microbial load were analyzed in terms of heterotrophic plate count (HPC), epifluorescence microscopy, carbohydrate quantity, total and free ATP concentration and compared material dependence. Kruskal-Wallis statistical analysis revealed that there were not significant differences between the tested slide materials according to HPC, DAPI-CTC staining, and ATP measurement. The ATP measurement together with fluorescence staining reflected the changes in the biofilms more distinctly than the HPC.

Keywords: Biofilm, cooling tower, stainless steel, ATP, DAPI-CTC double staining
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Model bir soğutma kulesi sisteminde polivinil klorid, paslanmaz çelik ve cam yüzeyler üzerindeki biyofilmle ilişkili bakterilerin farklı mikrobiyolojik yöntemlerle incelenmesi

Özet

Soğutma kuleleri birçok endüstriyel sürecin parçasıdır. Soğutma suyu sistemindeki koşullar mikrobiyal gelişim ve biyofilm oluşumu için ideal bir ortam sağlar. Tamamlama suyu veya havadan sisteme giren mikroorganizmalar su ve kısıtlı besin içeren soğutma kulesinin iç yüzeylerinde biyofilm tabakası oluştururlar. Soğutma kulesi sistemlerinde biyofilm oluşumu, sistemin çalışma koşulları ve kamu sağlığı nedenleri ile istenmeyen bir durumdur. Bu nedenle mikrobiyal yük takip edilmeli ve kontrol altında tutulmalıdır. Çalışmada su ve biyofilmle ilişkili mikrobiyal yük heterotrofik plak sayım, epifloresan mikroskobi, karbonhidrat miktarı, toplam ve serbest ATP konsantrasyonu açısından incelenmiş ve materyaller kıyaslanmıştır. Kruskal-Wallis istatistik analizine göre test edilen malzemeler arasında heterotrofik plak sayım, epifloresan mikroskobi, toplam ve serbest ATP konsantrasyonu açısından önemli bir fark tespit edilmemiştir. Floresan boyama ve ATP ölçümü tekniklerinin birlikte kullanımı biyofilmdeki değişiklikleri heterotrofik plak sayımdan daha net yansıtmaktadır.

Anahtar Kelimeler: Biyofilm, soğutma kulesi, paslanmaz çelik, ATP, DAPI-CTC boyama

Introduction

Cooling tower systems are used to dispose of excess heat generated in industrial processes such as power plants, air-conditioning systems, telecommunication devices, and oil refineries. In a cooling tower, process water is sprayed over packing material (such as polypropylene, polyvinyl chloride, polyethylene) and cools down by evaporation of water. The residual concentrated process water is collected in the basin of the cooling tower for reuse. The loss of water through evaporation and excess concentration of minerals is replaced by the intake of make-up water and the discharge (blowdown) of some water from the cooling system (Meesters et al. 2003). Microorganisms which enter via makeup water or air to the system form a biofilm layer on the inside surfaces of cooling tower which contain water and limited nutrients.

Biofilms are the product of adhesion and growth of microorganisms on surfaces (Allison 2003). In biofilm conditions, microorganism cells are embedded in a thick mucilaginous matrix of extracellular polymeric substances (EPS) which include polysaccharides, proteins, and nucleic acids. On the one hand, biofilm layer act as biological filters, provides mechanical stability and also protects its inhabitants from physico-chemical alterations occurring in the bulk water phase (Costerton 1999; Schwartz et al. 2003). On the other hand, biofilms in cooling towers lead to many undesired conditions such as equipment damage through corrosion, decreased energy efficiency due to increased hydraulic pressure (pumping costs), local blocking of cooling towers and increased heat transfer resistance. Furthermore, biofouling is a source for the accumulation of hygienically relevant pathogenic bacteria especially *Legionella pneumophila*, which can be spread into the environment through aerosols generated in the cooling tower (Meesters et al. 2003; Schwartz et al. 2003; Sanli-Yurudu et al. 2007).

The formation of biofilm depends on many factors prevailing in the water system, e.g. types of surface materials, water temperature, pH, microbial quality of intake water, concentration and quality of nutrients, the presence of a disinfectant residual (Zacheus et al. 2000; Momba and Binda 2002; Momba and Makala 2004; Türetgen and Cotuk 2007; Kimiran-Erdem et al. 2008).

Because of the economic loss, operational and public health reasons, both bulk water and biofilm layer of cooling towers' should be monitored and control in view of microbial burden.

To assess microbial activity different techniques such as conventional plate count, light, electron and epifluorescence microscopy, fluorescence antibody staining techniques, measurement of ATP concentrations, biochemical parameters, cell wall components and respiration rates have been used.

Since the characteristics of the surface material greatly influence the densities of biofilm formation and monitoring of microbial burden by effective techniques would be beneficial, the study was planned to quantify microbial load of both bulk water and biofilm on different surfaces (stainless steel, glass, PVC) in model cooling system. In quantification of microbial load, samples were analyzed in terms of heterotrophic plate count, epifluorescence microscopy, carbohydrate quantity, total and free ATP concentration.

Materials and methods

Model system

The experimental study was performed using a 100-liter polypropylene laboratory scale recirculating cooling tower model system under constant hydraulic conditions, to simulate cooling tower installations. For modeling microbial flora of cooling tower, real cooling tower system water (at 35ºC) was inoculated to the model system at the beginning of the experiment and a real cooling tower nozzle has adapted to provide dispersion of water on slide surfaces. Model system was equipped with a recirculation pump (550 W, 40 $l.min^{-1}$, Pedrollo, Italy) in the basin and a heater (AT–100, 100 W, Atman, Germany) to facilitate evaporation and provide accurate temperature control. Throughout the experiment (180 days), the water temperature was kept constant at 37ºC and cover lid had openings to ensure fresh air and daylight entry (Figure 1). Public potable water was used to replenish water lost by evaporation and blowdown (partial draining).

No chemicals (disinfectant, pH regulators or anti-scaling agents) were added to the system in order to exclude their possible negative effects (such as their disinfecting effect) on the microorganisms and biofilm formation. The water samples were analyzed monthly for various physico-chemical parameters such as pH, conductivity (WTW LF 95 Conductivity meter, Germany), total dissolved solids (TDS) and dissolved oxygen.

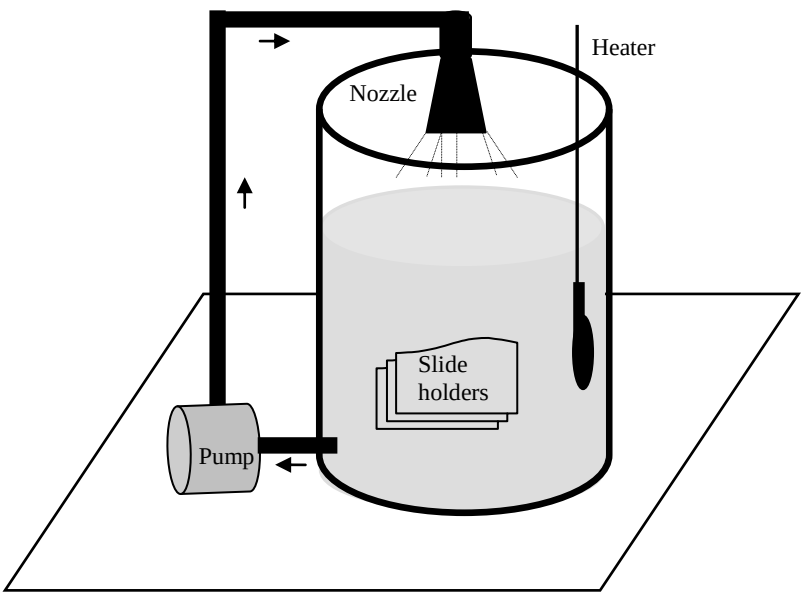


Figure 1. The schematic diagram of recirculating cooling tower model system, arrows indicating the flow direction.

Test slides

Stainless steel slides (304) (SS) were prepared in 25x60x0.8 mm, glass (G) and polyvinyl chloride (PVC) slides 25x76x1 mm dimensions by laser cutting. Before placed to the model system all slides cleaned using a neutral detergent (1% Triton) (Miettinen and Schaule 2003) and after rinsing them with tap water, degreasing was carried out in acetone for 3 min by ultrasonicator. Then, rinsed sterile distilled water, dried at 60ºC both slide surfaces was disinfected by transilluminator (TI–100, Tomy Seiko Co. Ltd, Japan) for 12 hours (Miyanaga et al. 2007; Sanli-Yurudu et al. 2007).

After sterilization and testing for the presence of any residual detergent, the slides were placed into the vertically into slide–holders situated in the water basins.

Microbial analysis

Both water and biofilm samples were collected from the model system monthly. Samples were analyzed in terms of heterotrophic plate count (HPC), epifluorescence microscopy, carbohydrate quantity, total and free ATP concentration.

To prepare biofilm samples three slides of each material were removed aseptically from the basin, dip-rinsed in sterile phosphate buffer to remove unattached cells. Biofilms on surfaces were scraped by sterile scalpel and suspended in 10 ml sterile tap water by vortexing (60 s) and stomaching (60 s) (Gagnon and Slawson 1999). Biofilm homogenates were then serially diluted from 10^{-1} to 10^{-5}.

In bulk water samples, 1 L model system water was taken aseptically with sterile bottle. Samples were then serially diluted from 10^{-1} to 10^{-5} with sterile tap water.

For heterotrophic plate count (HPC), both diluted biofilm homogenates and bulk water were spread-plated (0.1 mL) onto R2A agar (Oxoid, UK) plates and incubated at 28 °C for 10 days. After incubation colonies were counted with a Colony Counter Device (aCOLyte Super Colony Counter, Synbiosis). HPC determinations were done in triplicate (Reasoner and Geldreich 1985).

Epifluorescence microscopy was used to evaluate the number of total and respiring bacterial cells in biofilms and bulk water. The tetrazolium salt 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Sigma-Aldrich) was used in conjunction with DNA-binding 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) fluorochrome to differentiate metabolically active cells from dead cells (Rodriguez et al. 1992; Johansen et al. 1997).

900 µl of both bulk water and homogenized biofilm samples were incubated with aliquots of a 50 mM CTC redox dye solution (5 mM final concentration of dye) in the dark at 28 °C for 4 h. Then, samples were counterstained with 1.0 $\mu g.ml^{-1}$ DAPI for 1 h. After incubation, samples were filtered by vacuum filtration onto black 0.2 µm pore size polycarbonate filters (Millipore, USA) (Schwartz et al. 2003; Türetgen 2008). The air-dried filters were mounted on a glass microscope slide with non-fluorescent immersion oil below the filter and coverslipped. Microscopic examinations were performed A Nikon 80i microscope equipped with 100 W mercury lamp. For statistical evaluation, the numbers of microorganisms were estimated from counts of 20 randomly chosen fields (at x 1,000) per sample. Following the manufacturer's instructions, all red signals were considered live cell while blue signals were considered dead. The number of microorganisms present in 1 ml of sample is calculated by applying the following conversion formula:

$$N = \frac{S \times n}{C \times V} \times D$$

where *N*, number of microorganisms per milliliter; *S*, real area of filtration; *n*, average number of microorganisms per field of vision; *C*, real area of microscopic range; *V*, volume of filtered sample; *D*, sample dilution.

Three slides of each material were removed and EPS extraction was performed according to the method of Zhang et al. (1999). The carbohydrate quantity of EPS was measured colorimetrically using the phenol/sulphuric-acid method (Dubois et al. 1956). Standard aqueous solutions of glucose (10–100 $mg.L^{-1}$) were used for instrument calibration.

Both bulk water and homogenized biofilm samples were taken using total and free ATP swabs. The swab systems, which held the reagents in a specialized container (luciferin and luciferase enzyme-substrate) necessary for testing, extract ATP from microbial cells. Released ATP reacts with luciferin and luciferase, within a few seconds, the entire contraption is inserted into the luminometer (Uni-Lite, Biotrace) and the relative light units (RLUs) are returned. (Davenport et al. 2002;

Kooij and Veenendaal 2002; Mattila 2002; Lim et al. 2005). The microbial ATP concentration was determined by the difference between total and free ATP.

Statistical analysis

The data were analysed using the Statistical Package for the Social Sciences (SPSS). The mean and standard deviation (SD) within samples were calculated in all cases. Kruskal-Wallis non-parametric tests were used whether the differences between the tested slide materials under the same conditions could be considered significant. Statistical calculations were based on confidence level equal higher than 95% (differences were considered significantly different when P).

Results

Physico-chemical and microbiological parameters of real cooling tower system water

Determined physico-chemical and microbiological parameters of real cooling tower system water can be seen in Table 1.

Physico-chemical parameters of model system water

pH, dissolved oxygen (DO), total dissolved solids (TDS) and conductivity values of simulated recirculating cooling tower system for six months were presented in Table 2.

Table 1. Some physico-chemical and microbiological characteristics of real cooling tower system water

Parameters	
Temperature (ºC)	35
TDS (mg.L^{-1})	2570
pH	8.41
Dissolved oxygen (mg.L^{-1})	6.95
Total ATP (RLU.ml^{-1})	1690
Free ATP (RLU.ml^{-1})	712
Heterotrophic plate counts (log cfu.ml^{-1})	5.18
Total microorganisms count (cell.ml^{-1})	2.86x10^{6}
Live microorganisms count (cell.ml^{-1})	2.13x10^{6}
Respiration activity (%)	74.47

cfu: colony forming unit.

Table 2. pH, dissolved oxygen (DO), total dissolved solids (TDS) and conductivity values of simulated recirculating cooling tower system for six months.

Months	pH	DO (mg.L^{-1})	TDS (mg.L^{-1})	Conductivity (µS.cm^{-1})
0.day	8.53	6.06	613	0.87
1	8.25	5.80	555	0.86
2	8.17	5.98	645	0.89
3	8.34	5.44	673	0.91
4	8.24	5.35	648	0.89
5	8.26	7.04	638	0.88
6	8.29	6.96	631	0.88

µS: MikroSiemens

Heterotrophic Plate Counts of Slides

HPC values of bulk water varied between 5.1- 6.09 log cfu.ml^{-1} (Figure 2). HPC values on SS, G and PVC slides reached maximum values 4.65, 5.02 and 5.06 log cfu.cm^{-2} respectively. It has been observed that bulk water HPC counts were generally higher than their sessile counterparts on the surfaces of tested materials.

Epifluorescence microscopy

While respiration activity of bulk water decreased at the 2nd month, it was increased at the 3rd month however then it decreased again at the 4th month. Respiration activity of bulk water have reached maximum of a 92.5% at the 6th month (Table 3). Similarly, at the same biofilm age, respiration activity value reached maximum level on G and PVC slide surfaces 87.5 and 73.1%, respectively (Table 3). Respiration activity of biofilm samples from SS slide surfaces was found minimum level at the 1st month (46.99%) and maximum level at the 4th month (99.45%) (Table 3).

The epifluorescence microscopy photo-graphs of the highest respiration activity of biofilm samples on SS (at the 6th month), G (at the 4th month) and PVC surfaces (at the 4th month) can be seen Figure 3.

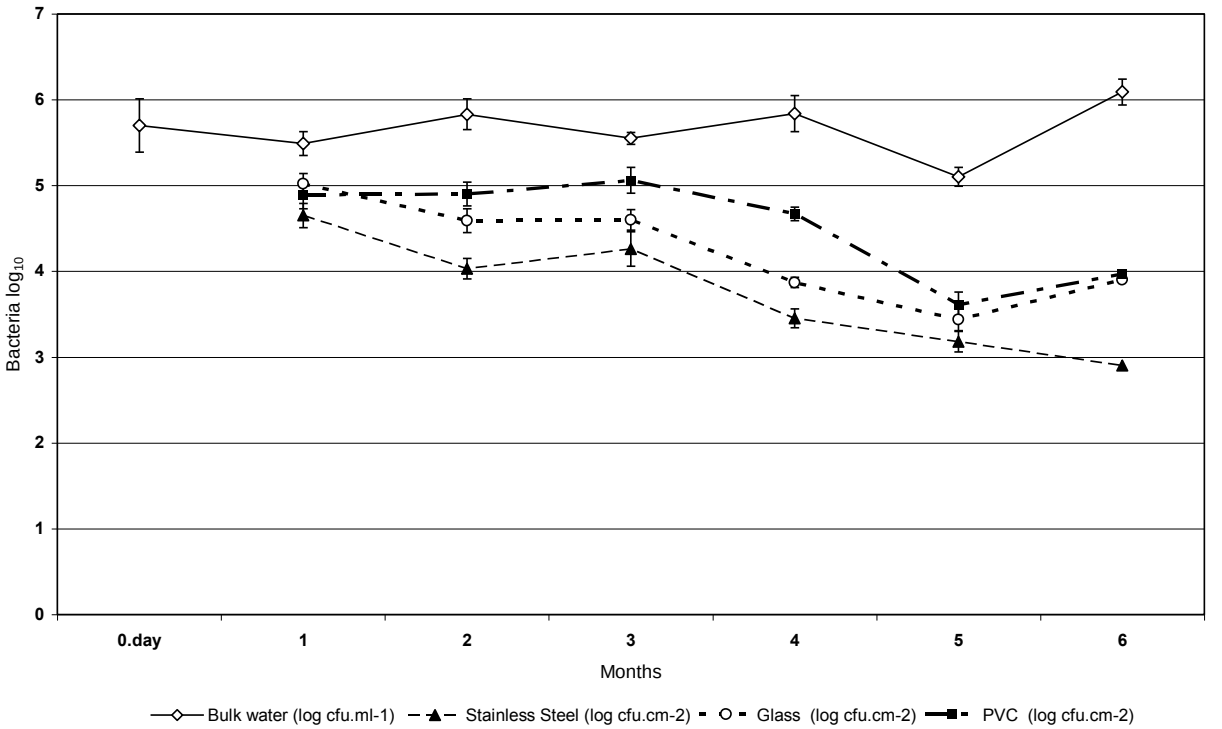
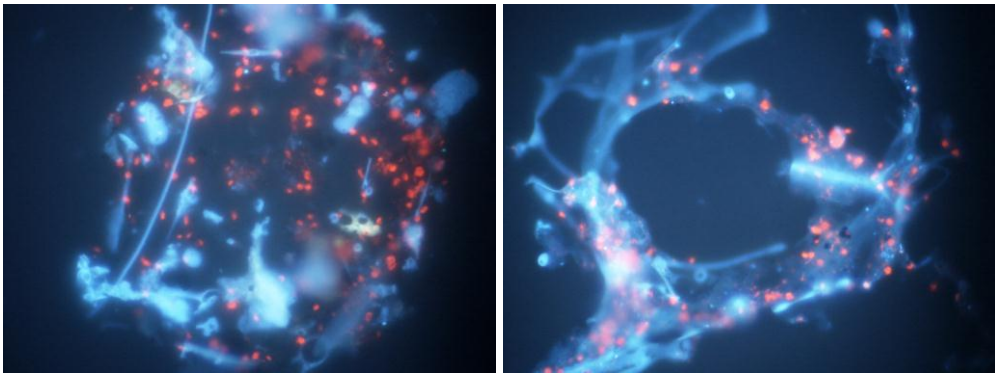


Figure 2. Heterotrophic plate counts of bulk water and biofilm on stainless steel, glass, polyvinyl chloride surfaces for 6 months. cfu: colony forming unit.

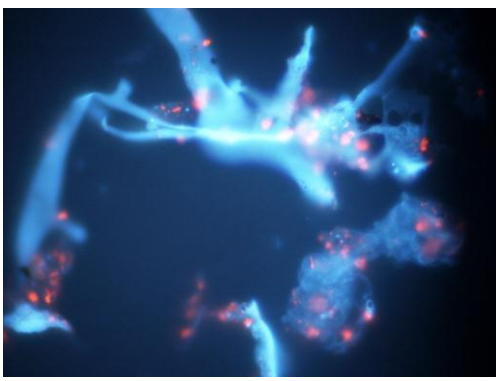
Table 3. Total (live + dead) and live microorganisms count from stainless steel, glass, PVC slides (cell cm^{-2}) and bulk water (cell mL^{-1}) for 6 months period. Values are arithmetical means of 3 replicates and represent the standard deviation of the mean.

		SS	G	PVC	Bulk Water
MONTHS					
0.day	**Total microorganisms count[a]**	-	-	-	9.99 (±0.13) x 10^6
	Live microorganisms count[b]	-	-	-	4.91 (±0.13) x 10^6
	Respiration activity (%)	-	-	-	49.15
1	**Total microorganisms count**	2.31 (±0.11) x 10^6	1.24 (±0.08) x 10^5	1.42 (±0.13) x 10^5	1.08 (±0.09) x 10^6
	Live microorganisms count	1.08 (±0.15) x 10^6	5.79 (±0.14) x 10^4	4.45 (±0.19) x 10^4	9.74 (±0.15) x 10^5
	Respiration activity (%)	46.99	46.42	31.25	90.02
2	**Total microorganisms count**	8.27(±0.12) x 10^5	2.49 (±0.18) x 10^5	1.74 (±0.14) x 10^5	1.82 (±0.2) x 10^6
	Live microorganisms count	4.92 (±0.16) x 10^5	1.33 (±0.13) x 10^5	9.8 (±0.06) x 10^4	1.21 (±0.16) x 10^5
	Respiration activity (%)	59.55	53.57	56.17	6.66
3	**Total microorganisms count**	8.28 (±0.11) x 10^5	7.57 (±0.17) x 10^5	1.06 (±0.15) x 10^6	1.32 (±0.12) x 10^6
	Live microorganisms count	6.46 (±0.18) x 10^5	1.64 (±0.19) x 10^5	3.34 (±0.12) x 10^5	1.8 (±0.13) x 10^5
	Respiration activity (%)	78.03	21.76	31.38	13.65
4	**Total microorganisms count**	7.29 (±0.12) x 10^5	6.68 (±0.09) x 10^5	6.06 (±0.14) x 10^5	8.57 (±0.11) x 10^6
	Live microorganisms count	7.25 (±0.08) x 10^5	3.16 (±0.13) x 10^5	1.91 (±0.16) x 10^5	5.41 (±0.17) x 10^4
	Respiration activity (%)	99.45	74.73	31.61	6.31
5	**Total microorganisms count**	2.13(±0.07) x 10^5	4.99 (±0.06) x 10^5	4.08 (±0.09) x 10^5	4.97 (±0.14) x 10^5
	Live microorganisms count	1.55 (±0.12) x 10^5	3.92 (±0.15) x 10^5	1.74 (±0.12) x 10^5	2.14 (±0.12) x 10^5
	Respiration activity (%)	72.55	78.57	42.64	43.05
6	**Total microorganisms count**	5.50 (±0.16) x 10^5	7.6 (±0.07) x 10^5	1.29 (±0.19) x 10^6	1.55 (±0.08) x 10^5
	Live microorganisms count	5.25 (±0.15) x 10^5	6.65 (±0.11) x 10^5	9.44 (±0.03) x 10^5	1.44 (±0.1) x 10^5
	Respiration activity (%)	94.84	87.50	73.10	92.5

SS: stainless steel, G: glass, PVC: polyvinyl chloride
[a]Total microorganisms counts determined by DAPI + CTC staining
[b]Live microorganisms counts determined by CTC staining



A- SS (At the 4th month) B- G (At the 6th month)



C- PVC (At the 6th month)

Figure 3. Epifluorescence microscopy photographs of the highest respiration activity of biofilm samples on stainless steel, glass polyvinyl chloride surfaces.

Carbohydrate Analysis

For 4 months, carbohydrate quantity on SS slides increased gradually and then it was decreased at the 5th month. On the other hand carbohydrate quantity on G and PVC surfaces increased at the first two months, after that the carbohydrate content of G slides increased and reached maximum level at the 6th month (Figure 4).

When carbohydrate contents of surface material were compared carbohydrate quantities were in order of SS > G > PVC (Figure 4).

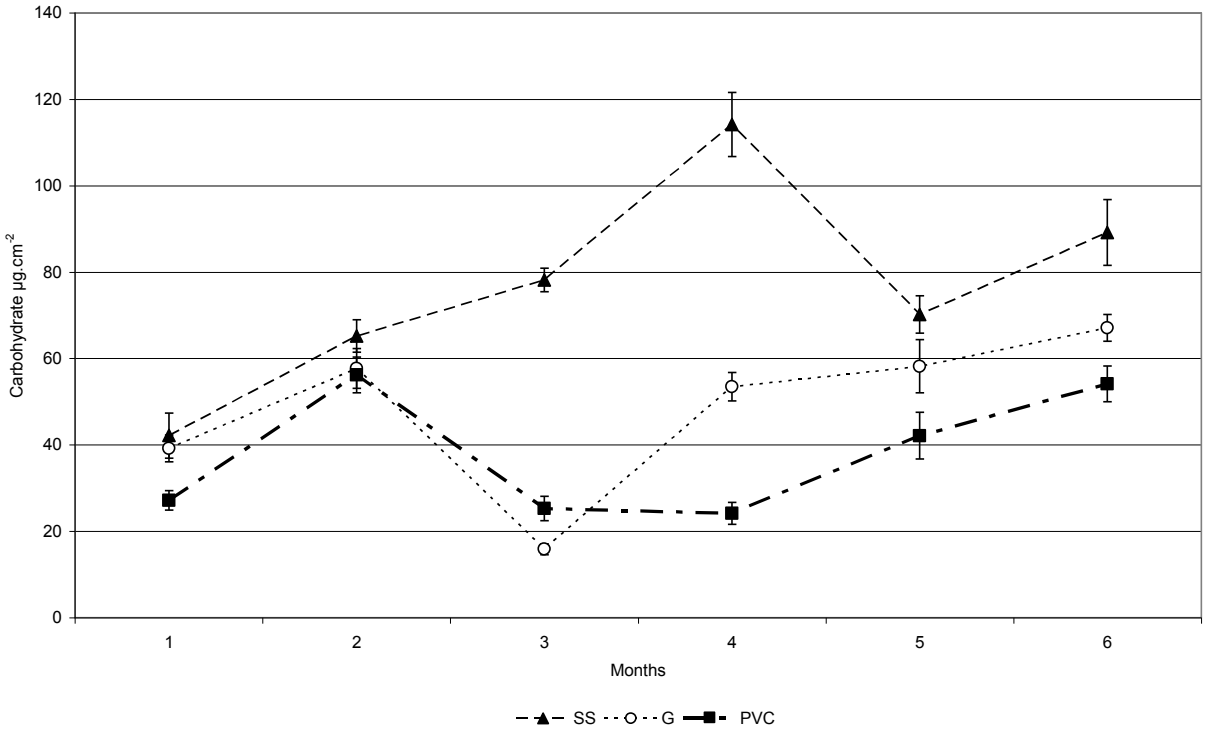


Figure 4. The carbohydrate quantity of biofilm on stainless steel, glass, polyvinyl chloride surfaces for 6 months.

ATP measurement

Biofilm samples contained more total and free ATP levels compared to the bulk water (Table 4). Calculated microbial ATP concentration values, which was mentioned in materials and methods' section, showed similar increase and decrease with respiration activity results for both bulk water and biofilm samples (Table 3 and Table 4). Microbial ATP concentrations were correlated with respiration activity ($r = 0.93$, $P < 0.05$) and carbohydrate contents ($r = 0.88$, $P < 0.05$).

Furthermore the results obtained by ATP-bioluminescence technique suggested that model system water was not in a satisfactory hygienic condition, with values above 300 RLU for total ATP, and in warning conditions, between 150 and 300 RLU for free and microbial ATP (Table 4).

Table 4. Total and free ATP levels from stainless steel, glass, PVC slides (RLU cm^{-2}) and bulk water (RLU mL^{-1}) for 6 months period. Values are arithmetical mean of three repetitions

Months		Total ATP	Free ATP
0. day	**Bulk water**	145	86
1	**SS**	1211	736
	G	1990	1600
	PVC	1480	1140
	Bulk water	1927	278
2	**SS**	341	118
	G	1190	680
	PVC	3080	2540
	Bulk water	178	61
3	**SS**	1328	133
	G	1170	910
	PVC	578	247
	Bulk water	221	49
4	**SS**	2331	201
	G	960	160
	PVC	616	251
	Bulk water	198	55
5.	**SS**	1051	157
	G	1154	184
	PVC	737	251
	Bulk water	255	140
6	**SS**	1868	160
	G	1372	162
	PVC	1380	690
	Bulk water	1669	548

Relative Luminescence Units: RLU
SS: stainless steel, G: glass, PVC: polyvinyl chloride

Discussion

Model system which was used in this study has been run to the nearest operating conditions of full-scale system. Thus, model system was similar to the full-scale system, which was used for modeling microbial flora of cooling tower, in terms of temperature, pH, dissolved oxygen values. In cooling systems, diminishing water by evaporation and discharge is completed with make-up water in order to keep the concentration TDS (total dissolved solids). Increasing of the TDS causes an elevation of the pH value and electrolytic property of the tower water and thus, an increase in the corrosiveness of water (EPA 2003). Model and full-scale systems were also similar because of their differences from chemical properties of tap water (make-up water). Mains water pH and TDS values were extremely low, while the real and model system water values were very high. On the other hand, TDS values of real system were much higher than model system, this difference was probably raised from corrosion of cooling tower material (carbon steel, which is very susceptible to corrosion) by cooling water.

Optimum growth temperature is about 40 ºC for many bacteria in cooling water and temperature measurements is taken close to the this degree in industrial cooling systems especially in summer times (Melo ve Bott 1997). It has been stated that biofilm formation is restricted by increasing of the water flow rate which cause detachment of biofilm and a decrease in biofilm thickness in system. (Cloete

2003). Considering these factors, model system was kept at 37 ºC and a real cooling tower nozzle has adapted to provide dispersion of water on slide surfaces. Indeed, when HPC of bulk water and biofilm samples were analyzed during 6 months' study, unlike other biofilm studies HPC values were found higher in bulk water than in biofilm samples at each sampling time. It has been thought that this difference might be arisen from the nozzle adaptation which cause speed-up of biofilm detachment by hit of water to the surfaces.

Biofilm associated microorganisms, which can adapt very quickly to changing environmental conditions, lead to significant economic loss. In cooling water systems, which are necessary parts of the many industrial processes, biofilm layer cause acceleration of the metallic corrosion, reduction of heat transfer capacity, increase of fluid friction resistance and thus energy loss. Furthermore, biofouling leads to congestions in certain areas and increase of the pumping costs (Melo ve Bott 1997; Cloete 2003)

One of the crucial points in biofilm formation is the type of surface material. Studies have indicated that each material does not allow biofilm formation equally (Melo and Bott 1997; Türetgen and Cotuk 2007; Kimiran-Erdem et al. 2008). It has been stated that one of the most appropriate material for biofilm formation is latex and followed by ethylene-propylene, polyethylene, mild steel, rigid PVC, chlorinated PVC, polypropylene, stainless steel and glass, respectively (Rogers et al. 1994a).

Cooling towers are usually made of metals and to cool water a packing material is used. Packing material fill is composed of thin sheets of plastic material to create increased surface area upon which the water flows. Since stainless steel is frequently used in the construction of cooling towers due to its resistance to corrosion, PVC is commonly used as fill material in many cooling tower systems and glass represents a control material due to its non-toxic and inert chemical characteristics, they were chosen for this study.

Control and monitor of microbial contamination is one of the most critical functions of a water microbiologist. Maintaining control over microbial contamination requires an accurate assessment of the amount of contamination present. To determine microbial load in cooling towers most frequently used method is conventional plate count. (Cloete 2003). One of the most important problem in this method, residual biocide in the sample can continue to kill bacteria until plating time and thus cause false and/or low results. Another problem is inhibition of the bacterial metabolism as a result of differences in nutrient conditions and therefore they cannot grow in culture. Heterotrophic plate count method allows growth of only a small, variable percentage of the organisms (< 10%) present in a water system on standard microbiological media and obtaining of the results require 24-240 hours. Alternatively, measurement of ATP (adenosine triphosphate) which is a criterion of metabolically active biomass concentration and determinations of total and metabolically active microorganisms' populations with special fluorescent dyes have been suggested. (Davenport et al. 2002; Mattila 2002; Cloete 2003; Lim et al. 2005)

In the ATP-luminometric test, luciferase (firefly enzyme) in the presence of luciferin substrate, oxygen and magnesium ions catalyzes conversion of chemical energy of ATP into light through oxidation-reduction reaction. The quantity of generated light is directly proportional to the amount of ATP present, thus, the light units can be measured to estimate the biomass of cells in a sample. ATP test have several advantages over traditional plating methods in that they require less than a minute to perform and signal is obtained from every cell in the system.

Total ATP tests measure both the ATP that is bound up within living cells as well as ATP that is floating free in the water. Free ATP tests measure only the ATP floating free outside of living cells. The microbial ATP concentration was determined by the difference between total and free ATP.

In clean cooling tower conditions, < 10^4 cfu.ml^{-1} heterotrophic bacterial count and < 300 RLU ATP concentration indicate a successful control program. In the present study, HPC values of bulk water > 10^4 cfu.ml^{-1} was found during the 6 months experiment period. On the other hand, ATP concentrations were found > 300 RLU only at 1st and 6th months in bulk water, however results should be compared with also epifluorescence microscopy results.

In the current study, DAPI and CTC fluorescent dyes were used for epifluorescence microscopy. DAPI targets DNA in both dead-live all the cells, inducing blue fluorescent and it is suitable for the enumeration of total cell count. Oxidized CTC is nearly colorless and is nonfluorescent; however, the compound is readily reduced via electron transport activity during cellular respiration and forms red fluorescent, insoluble CTC-formazan and thus allows for display only respiring cells. Therefore, microorganisms which can not growth in traditional method will determine easily and more accurate results can be obtained by ATP technique and fluorescence staining. In our experiments, in ATP tests and fluorescence staining very high correlation was determined ($r = 0.93$, $P < 0.05$) (Table 3 and 4). Carbohydrate contents on surfaces were also correlated with ATP concentrations ($r= 0.88$, $P < 0.05$) and fluorescence staining ($r= 0.93$, $P < 0.05$) (Figure 4, Table 3 and 4). Results of the HPC were smaller than the live microorganism counts found by the CTC staining technique (Figure 2 and Table 4) and HPC values and other techniques which used in this study did not correlated (Figure 2 and 4; Table 3 and 4).

The ATP measurement together with fluorescence staining reflected the changes in the biofilms more distinctly than the HPC. According to these results, a combination of ATP technique, carbohydrate determination and fluorescence staining may be useful in the microbiological risk assessment of system pollution.

Rogers et al. (1994b) studied the influence of the plumbing materials on biofilm formation and growth of *L. pneumophila* and total flora in potable water systems over 28 days. They found the lowest total flora on stainless steel according to bacterial culture counts. In another study, Türetgen and Cotuk (2007) investigated biofilm associated *L. pneumophila* and heterotrophic bacteria in terms of material dependence, and found that HPC values was in order of SS > G > PVC. On the other hand, Zacheus et al. (2000) found that there were no clear differences in the formation of biofilm on different surfaces (polyvinyl chloride, polyethylene and stainless steel) according to heterotrophic bacterial counts, organic carbon measurement and acridine orange staining results.

In the current study, heterotrophic bacterial counts on PVC were found significantly higher than that on other two materials at each sampling time (Figure 2), while stainless steel was the most colonized surface in view of microbial ATP and fluorescence staining (Table 3 and 4). However, it should be taken into consideration that Kruskal-Wallis non-parametric analysis revealed that there were not significant differences between the tested slide materials according to HPC, DAPI-CTC staining, and ATP measurement (asymptotic significance values 0.135, 0.075, 0.264, respectively). Our findings are coherent with Zacheus et al's results (2000).

The formation of biofilm is affected with various factors. One of those factors is the surface materials, which are used in water systems. The importance of surface material on organism numbers and evaluation of different the microbiological methods on biofilm determination, was substantiated in this research. Selection of the material will not guarantee absence of the waterborne pathogens. Appropriate material usage minimize the possibility of biofilm development thereby reduce associated illnesses risks and economic loss. It seems that plastic materials such as PVC may provide better cleaning as fill material of cooling towers.

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