Turkish Journal of Biology

Volume 34 | Number 4 Article 6

1-1-2010

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ESRA İLHAN SUNGUR

İRFAN TÜRETGEN

REZA JAVAHERDASHTI

AYŞIN ÇOTUK

Turk J Biol
34 (2010) 389-397

doi:10.3906/biy-0902-8

Monitoring and disinfection of biofilm-associated sulfate reducing bacteria on different substrata in a simulated recirculating cooling tower system

Esra İLHAN SUNGUR[1,*], İrfan TÜRETGEN[1], Reza JAVAHERDASHTI[2], Ayşın ÇOTUK[1]

[1]Faculty of Science, Department of Biology, İstanbul University, 34134 Vezneciler, İstanbul - TURKEY

[2]Dept. of Petroleum Eng., Curtin University Senior Corrosion Engineer (Extrin) and Visiting Senior Lecturer / Researcher, Perth, AUSTRALIA

Received: 05.02.2009

Abstract: Microbial biofilm and corrosion in cooling systems are the most common problems that damage expensive equipment, cause loss of production, and increase maintenance costs. Sulfate reducing bacteria were considered the major bacterial group involved in microbiologically influenced corrosion (MIC). We investigated the survival and enumeration of biofilm-associated SRB on coupons of galvanized steel, stainless steel, and copper, which are materials used in the manufacturing of cooling systems. We also investigated the effect of monochloromine on SRB as in mixed-species mature biofilms formed on coupons by simulating recirculating cooling water conditions, due to the better penetration feature in biofilms than the residual chlorine. It was concluded that SRB count increased with time in bulk water and the surfaces ($P < 0.01$). Experimental results supported by statistical analyses show that monochloromine is poorly effective on SRB colonies formed on galvanized and stainless steel surfaces.

Key words: Sulphate reducing bacteria, biofilm, cooling tower, galvanized steel, stainless steel, copper

Soğutma kulesi model sisteminde farklı tipte yüzeylerdeki biyofilmle ilişkili sülfat indirgeyen bakterilerin takibi ve dezenfeksiyonu

Özet: Soğutma kulelerinde mikrobiyal biyofilm ve korozyon ekipmana zarar veren, üretim kaybına yol açan ve bakım maliyetlerini artıran hususlardır. Sülfat indirgeyen bakteriler (SRB) mikrobiyolojik korozyona (MIC) yol açtığı bilinen ana bakteri grubu olarak bilinir. Bu çalışmada biyofilm tabakasında bulunan SRB'lerin dağılımı, soğutma kulesi yapımında sıkça kulanılan galvanizli çelik, paslanmaz çelik ve bakır kuponlar üzerinde incelenmiştir. Ayrıca, soğutma kulesi model sistemi koşullarında doğal olarak oluşturulmuş olgun biyofilm tabakası içinde bulunan SRB'lere karşı, biyofilm tabakasına serbest klordan daha iyi penetre olan monokloramin dezenfektanının etkisi de araştırılmıştır. Su fazında serbest yüzen ve kuponlar üzerinde bulunan SRB sayısının zamanla orantılı olarak anlamlı şekilde arttığı bulunmuştur ($P < 0,01$). Deney sonuçlarına göre galvanizli çelik ve paslanmaz çelik kuponlardaki SRB'lere karşı monokloramin dezenfektanının çok zayıf şekilde etkili olduğu görülmüştür.

Anahtar sözcükler: Sülfat indirgeyen bakteriler, biyofilm, soğutma kulesi, galvanizli çelik, paslanmaz çelik, bakır

Introduction

Cooling towers (CTs) are an integral part of any power plant. The conditions of CTs are very suitable for microbial growth and biofilm formation (1,2). Biofilms can harbor opportunistic pathogens and corrosion agents such as *Legionella pneumophila* and sulfate reducing bacteria (SRB), respectively (1). It has been reported that at some power plants up to 200 different forms of bacteria can be found (3).

A biofilm is a community of cells embedded in a thick mucilaginous matrix of extracellular polymeric substances (EPSs), which may consist of 90% or more of polysaccharides (4). Bacterial EPSs provide important functions for protection, survival, and dispersion of biofilm-associated microorganisms (5).

The formation of biofilms is a dynamic process that involves stages during which the bacteria attach themselves to the conditioned surface of the metal and start to build a living net that will trap both organic and non-organic matter (6). Biofilm formation and its contribution to accelerating corrosion have been discussed in detail elsewhere (7).

Microbial biofilm and corrosion in cooling systems are the most common problems that damage expensive equipment, cause loss of production, and increase maintenance costs (1,8). SRB were considered the major bacterial group involved in microbiologically influenced corrosion (9).

Microbiologically influenced corrosion (MIC) can be defined in many ways. However, all these definitions carry certain elements common to all of them. These elements are as follows (7):

1. MIC is an electrochemical process,
2. Microorganisms are capable of affecting the extent, severity, and course of corrosion,
3. In addition to the presence of microorganisms, an energy source, a carbon source, an electron donor, an electron acceptor, and water must be also present to initiate MIC.

Biocides are used to keep potentially pathogenic or hazardous bacteria under control and maintain biofouling at minimal levels in man-made water systems. Chlorine has been used as a popular disinfectant for controlling bacterial growth in drinking water and cooling towers (10); however, monochloramine was shown to be more stable than free chlorine, even at high temperatures and elevated pH values (11). It has been speculated that the lower sensitivity of biofilm bacteria to biocides is due to the physiological status of the cells in biofilm and also due to the lower permeability in biofilm matrix (12). So far, the impact of monochloramine on SRB has not been evaluated on biofilms.

The aims of the study were to compare the presence and affinity of SRB to different construction materials and also the efficacy of monochloramine (recommended dose) against mature biofilms formed in a model recirculating water system on different surfaces under identical conditions (13). For these purposes, survival and enumeration of biofilm-associated SRB and heterotrophic bacteria were investigated on galvanized steel, stainless steel, and copper coupons in a simulated recirculating cooling tower water system over 180 days. Carbohydrate quantity was analyzed to evaluate EPS quantity on substrata.

Materials and methods

Model system and surfaces

The experimental study was performed using a 100-L polypropylene laboratory-scale cooling tower model system running with 80-L bulk water under constant hydraulic conditions, which simulated the situation in a cooling tower. It 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 within the bulk water. The cover lid had openings to ensure fresh air and daylight entry (Figure 1). A supply of potable water was used to replenish the water lost through evaporation and blowdown (partial draining). Throughout the experiment, the water temperature was kept constant at 29 °C. Materials that are commonly used in the construction of the cooling tower systems were selected (stainless steel AISI 316-2B, galvanized steel EN 10142-Z 200 and copper Cu-DHP, ASTM B 280). The coupons (20 × 50 × 1 mm) were obtained from local producers and were washed with general purpose detergent (Henkel, Germany), rinsed with distilled water, immersed in 70% ethanol for 5 min, and air dried before use (14). Biofilms were

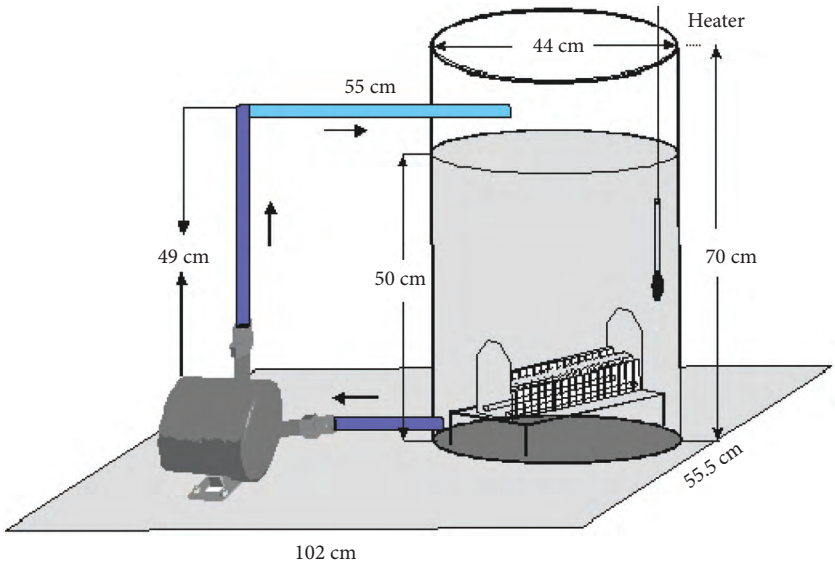

Figure 1. The schematic diagram of model recirculating water system, with arrows indicating the flow direction.

allowed to develop for 180 days on coupons within the aqueous phase of the system. Coupons were inserted vertically into coupon holders situated in the water basins. No chemicals (disinfectant, pH regulators, or anti-scaling agents) were added to the system so as to prevent their possible negative effects (such as their disinfecting effect) on the microorganisms and biofilm formation.

Bacterial analysis

Three coupons of each material, i.e. stainless steel, galvanized steel, and copper, were removed monthly from the basin and dip-rinsed in sterile phosphate buffer to remove unattached cells. Biofilms on surfaces were scraped using a sterile scalpel, suspended in 5 mL sterile phosphate buffer, and vortexed (Clifton Cyclone, England) for 60 s (15,16). The resulting suspensions were serially diluted to 10^{-7}.

SRB counts were determined by the most probable number (MPN) technique using Postgate's medium B with the following composition in anaerobic conditions: $C_3H_5O_3Na$ (3.5 g L^{-1}) (Sigma, UK), KH_2PO_4 (0.5 g L^{-1}) (Sigma, UK), NH_4Cl (1.0 g L^{-1}) (Sigma, UK), Ca_2SO_4 (1.0 g L^{-1}) (Sigma, UK), $MgSO_4 \cdot 7H_2O$ (2.0 g L^{-1}) (Sigma, UK), yeast extract (1.0 g L^{-1}) (Oxoid, UK), $C_6H_7O_6Na$ (0.1 g L^{-1}) (Sigma, UK), $C_2H_3O_2SNa$, (0.1 g L^{-1}) (Sigma, UK), $FeSO_4 \cdot 7H_2O$ (0.5 g L^{-1}) (Sigma, UK), $C_6H_5O_7Na_3$ (0.3 g L^{-1}) (Sigma, UK). pH was adjusted to 7.2 with 10% NaOH (Sigma, UK) (17,18). MPN tubes were incubated in the dark at 30 °C for 3 months (19). In each inoculated tube, the growth of sulfate reducers was indicated by the formation of a black FeS precipitate and by turbidity. The number of bottles containing the black precipitate was determined and SRB counts (mL^{-1}) were obtained from the MPN table (18). SRB analyses were carried out in duplicate.

For heterotrophic plate count (HPC), 10-fold diluted biofilm homogenates and bulk water were spread-plated (0.1 mL) onto R2A agar (Oxoid, UK) plates (20) and incubated at 28 °C for 10 days. After the incubation, the number of colonies was enumerated under a colony counter (BZG 30, WTW, Germany) and recorded as CFU mL^{-1}. HPC determinations were performed by triplicate analyses.

EPS extraction and carbohydrate analysis

Biofilm was removed from the surfaces with a sterile cotton swab and suspended in 10 mL of distilled water. The suspension was centrifuged at 6000 × *g* for 10 min. The liquid was decanted and the biomass pellet was then resuspended in a 10 mL of 8.5% NaCl solution containing 0.22% formaldehyde. The suspension was mixed in a vortex mixer (Fisons-WhirliMixer, SGP-202-010J) at high speed for 1 min

to recover the capsule-bound EPSs. The liquid from the 2 steps was combined and brought to a volume of 20 mL. The combined sample was centrifuged at 12,000 rpm (11,227 × *g*) for 30 min in a water cooled ultracentrifuge (Sanyo MS 60) with a TFT-6538 rotor (MSE). The supernatants were filtered through 0.22 μm filters (21). The amount of carbohydrate on the surfaces was quantitated colorimetrically using the phenol-sulfuric acid method (22) and with sucrose as the standard (23). Three standards of 0, 10, and 100 μg mL^{-1} of sucrose were run with each assay. Biofilm samples were put into sterile phosphate buffered saline; then 5% phenol solution was added to each sample. The mixtures were vortexed and placed in the dark for 60 min. Furthermore, 5 mL of concentrated sulfuric acid was added and absorbances were measured at 487 nm in a spectrophotometer (UV-150-02 Shimadzu, Japan). The results were expressed as μg mL^{-1} carbohydrate of biofilm on the coupons. The data represented the means of 3 measurements.

Biocide preparation

Biofilm was grown on galvanized steel, stainless steel, and copper surfaces for 6 months and then the model system was disinfected with monochloramine. Monochloramine stock solution was prepared by mixing 0.11 mg of ammonium chloride (Sigma, UK) with 100 ml of phosphate buffered saline and then 1 mL of 5% sodium hypochlorite (commercial bleach, Henkel, Turkey) was added. A residual of 1.5 ppm was maintained for 180 min and then neutralized with sodium thiosulfate. Log reductions of bacterial numbers on disinfectant-treated coupons were investigated after exposure and compared with an untreated control. Filter-sterilized chlorine-free drinking water was used to dilute the stock solution to obtain the desired working concentration. A monochloramine test kit (LP-8 LaMotte, US) was used to measure final concentrations as recommended by the manufacturer. All glassware used was chlorine-free.

Statistical analysis

Bacteria counts were $\log_{10}$ transformed and the standard deviation of the means was calculated. Differences between materials were tested by one-way ANOVA to compare variations in heterotrophic bacterial counts on the surfaces, followed by Student Newman Keul's post hoc test using SPSS. A nonparametric Kruskal-Wallis test was employed to detect statistically significant changes in the SRB counts. Statistical evaluation of the results was carried out by Spearman coefficient of rank order correlation.

Results and discussion

The colonization of the galvanized steel, stainless steel, and copper by microorganisms occurred from the beginning of the experiment, and biofilms that had heterogeneous structures formed on the 3 surfaces. Growth curves for sessile SRB and heterotrophic bacteria are shown in Figures 2a, 2b, and 2c.

HPC on the stainless steel and galvanized steel coupons reached the plateau phase (maximum numbers 204,173 and 501,187 cfu/cm^2 respectively) after the 3rd month (Figures 2a and 2b). Heterotrophic bacteria on copper were gradually increased through the first 5 months (maximum number 457,088 cfu/cm^2) (Figure 2c). The heterotrophic bacterial counts on galvanized steel were significantly higher than those on the other 2 materials ($P < 0.01$) (Figures 2a, 2b, and 2c).

The carbohydrate contents on surfaces were correlated with HPC counts ($R = 0.91$, $P < 0.05$) (Figures 2a, 2b, and 2c). After 4 months, carbohydrate quantity on stainless steel and copper coupons reached the plateau stage (Figures 2a and 2c), while carbohydrate quantity on galvanized steel coupons reached the plateau stage after 3 months (Figure 2b). No significant difference was found between the carbohydrate contents on the surfaces of stainless steel and copper coupons, while galvanized steel coupons contained more carbohydrate compared to the other 2 materials ($P < 0.01$).

The SRB count was seen to increase with time on stainless steel ($P < 0.01$), galvanized steel ($P < 0.05$), and copper surfaces ($P < 0.01$) (Figures 2a, 2b, and 2c). The cell concentrations of SRB on stainless steel, galvanized steel, and copper surfaces increased to a maximum of 1.4×10^6 cells/cm^2, 1.4×10^5 cells/cm^2, and 1.4×10^6 cells/cm^2 after 6 months, respectively. In addition, Kruskal-Wallis analysis revealed that there was no statistically significant difference in the mean of SRB counts obtained from stainless steel, galvanized steel, copper surfaces, and bulk water during the experiment.

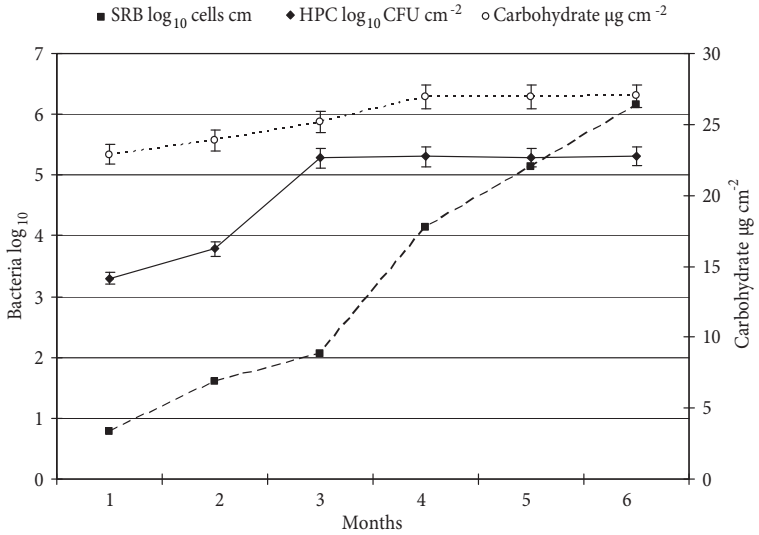

Figure 2a. The carbohydrate amount, heterotrophic bacteria, and SRB counts in biofilm on stainless steel surfaces over 6 months. Error bars represent the standard deviation. CFU: Colony forming unit, HPC: Heterotrophic plate count.

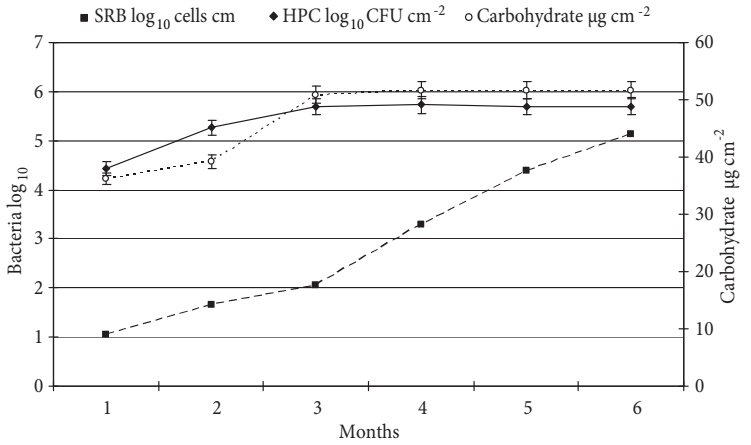

Figure 2b. The carbohydrate amount, heterotrophic bacteria, and SRB counts in biofilm on galvanized steel surfaces over 6 months. Error bars represent the standard deviation. CFU: Colony forming unit, HPC: Heterotrophic plate count.

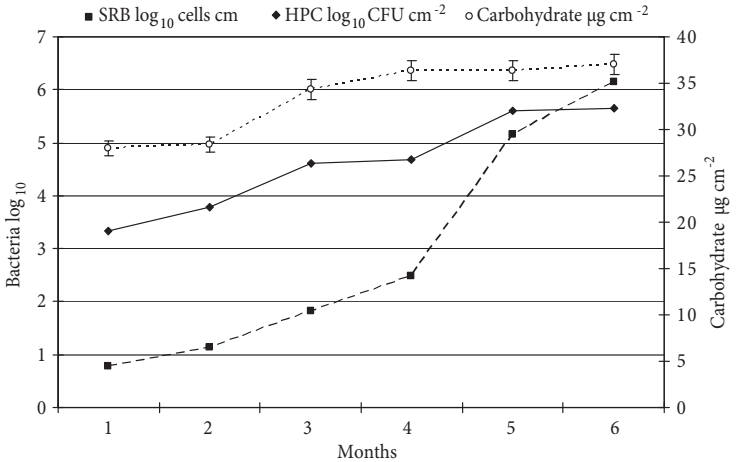

Figure 2c. The carbohydrate amount, heterotrophic bacteria, and SRB counts in biofilm on copper surfaces over 6 months. Error bars represent the standard deviation. CFU: Colony forming unit, HPC: Heterotrophic plate count.

It was concluded that SRB count increased over time in bulk water ($P < 0.01$) and reached a maximum of 1.4×10^7 cells mL^{-1}, while HPC in bulk water reached the plateau phase after the 3rd month (maximum count 3.4×10^4 CFU mL^{-1}) (Figure 3). It was also determined that there was a positive correlation between SRB count in bulk water and on surfaces ($P < 0.01$). Moreover, t test analysis revealed that aerobic heterotrophic bacteria counts in bulk water were higher than those in biofilms on copper surfaces during the experiment ($P < 0.01$). It was determined that there was a positive correlation between HPC in biofilms on the 3 surfaces and bulk water ($P < 0.01$).

After the disinfection process, log reduction of SRB counts (3 orders of magnitude) on the copper surface was significantly ($P < 0.01$) higher than that on stainless steel and galvanized steel surfaces. No significant biocidal activity was recorded against SRB on stainless steel and galvanized steel coupons (Figure 4a). However, Figure 4a shows that monochloramine was poorly effective against the SRB in biofilms on galvanized steel and stainless steel surfaces.

The pH increased gradually from an initial value of 6.58 to 8.42 during the experiment. The pH value was 8.48 at the moment of the biocide application.

A significant reduction in heterotrophic bacteria was recorded on stainless steel and copper surfaces, which was more than a 5 log reduction. However, no significant effect was found on the galvanized steel surface (Figure 4b).

The biocidal activity of monochloramine in bulk water resulted in a 2 log reduction against planktonic SRB and HPC after the exposure time (Figure 4a and 4b).

When microorganisms attach themselves to surfaces (or alternatively termed as "sessile" opposed to their planktonic state), a thin layer is formed called a "living film", or its more popular name, a biofilm (6).

Our findings suggest that aerobic heterotrophic bacteria and SRB were attached on galvanized steel and copper surfaces and observed in the biofilm although it is known that zinc and copper have toxic effects on a variety of microorganisms (24-28). İlhan-Sungur et al. (29) reported a similar result with respect to the attachment of SRB to galvanized steel. The nonparametric Kruskal-Wallis test revealed that there was no statistically significant difference in the mean of SRB counts obtained from stainless steel, galvanized steel, and copper surfaces without disinfection. On the other hand, Lin et al. (26) concluded that high pH in water may compromise the

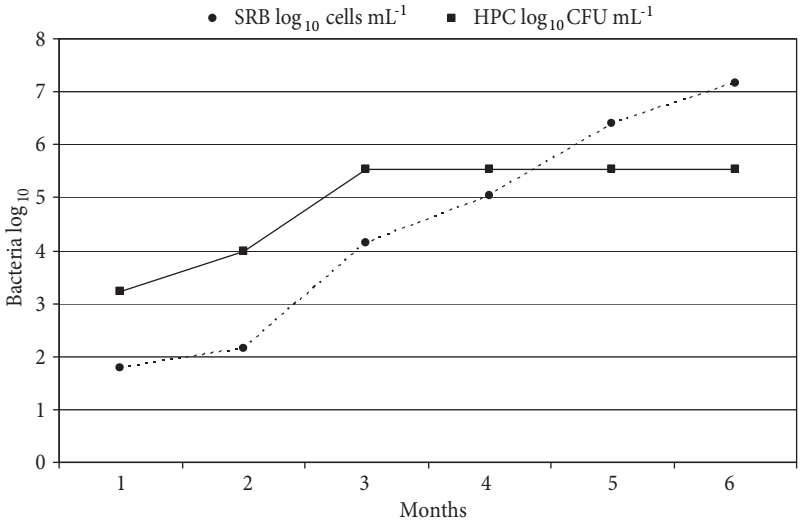

Figure 3. Heterotrophic bacteria and SRB counts in bulk water over 6 months. Error bars represent the standard deviation. CFU: Colony forming unit, HPC: Heterotrophic plate count.

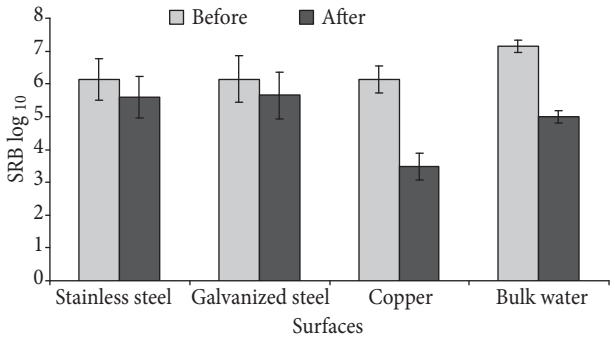

Figure 4a. SRB counts on surfaces (cell cm^{-2}) and bulk water (cell mL^{-1}) before and after disinfection at the end of the 6-month period. Values are arithmetical means of 2 replicates and error bars represent the standard deviation of the mean.

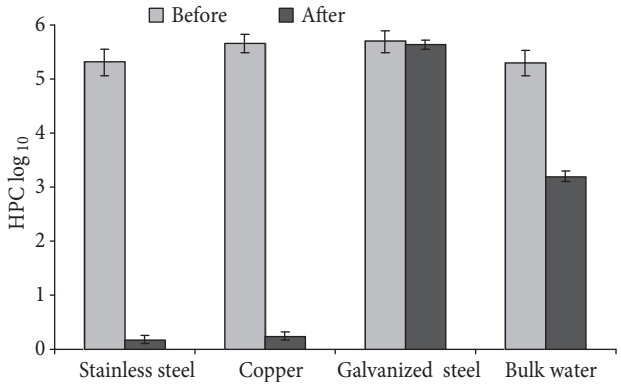

Figure 4b. HPC counts on surfaces (CFU cm^{-2}) and bulk water (CFU mL^{-1}) before and after disinfection at the end of the 6-month period. Values are arithmetical means of 3 replicates and error bars represent the standard deviation of the mean.

efficacy of copper ions. A similar aspect is experienced in chlorination; high pH values affected the efficacy of chlorination negatively (11).

Biofilm reached a steady-state phase in which the detachment–multiplication ratio is balanced with respect to the heterotrophic bacterial numbers after the third month (30).

The biofilm reproducibility of the surfaces taken from the model system was compared with the full-scale cooling tower system in a previous work (11), to reveal how representative the model system was. No significant difference was found in terms of HPC counts and reproducibility from tested coupons in the full-scale and model system, which was validated by a t test of the biofilm densities on coupons of 2 systems ($P < 0.05$) (11).

Extracellular polymeric substances mainly consist of carbohydrates, therefore measured to reflect the EPS matrix on surfaces. During the experiment, the correlation between the measured HPC and carbohydrate quantity was positive and significant ($P < 0.05$). The correlation reflects that increasing sessile cells are enclosed in very elaborate EPS structures.

As biofilm forms and thickens and reaches a certain thickness (that for some cases in aerobic environments has been reported as thin as 12 microns to make the spot anaerobic), it starts to act as a diffusion barrier (31). In this way, the transport of chemicals (including biocidal elements) may become restricted. That is the reason that some of the biocides may not be able to reach lower regions of the biofilm. Biocides are less effective on bacteria within biofilm than their planktonic counterparts in bulk water (32-34). Our results show that the material type affects the biofilm architecture on different surfaces, which could explain the success of biocide penetration into biofilms on copper surfaces. On the other hand, it is already known that biofilms show different architectural characteristics due to different surfaces, flow regimes, or primary colonizers (35), and these various architectural patterns affect the penetration of biocides.

Although numerous experiments are now available demonstrating the effects of some biocides such as glutaraldehyde, isothiazolone, and formaldehyde on SRB (36-39), to date we have not been able to find any published studies about monochloromine application on SRB in a mixed-species mature biofilm. A previous study (11) showed that the disinfection of biofilms with 1.5 ppm monochloramine for 180 min resulted in 3 orders of magnitude of reduction of heterotrophic bacteria within biofilm on the cooling tower surfaces. Our study showed that monochloromine is significantly ineffective against SRB on galvanized steel and stainless steel surfaces ($P < 0.01$); however, it can be concluded from Figure 4a that monochloramine is

poorly effective against SRB in biofilms on galvanized steel and stainless steel surfaces. Our findings suggest that if biofilm is formed and the sessile bacteria establish themselves within it, there will be basically little chance for the biocide (monochloromine in our study) to reach the bottom of the biofilm and eliminate it.

An immediate lesson from this part of the study, especially for industry, is to monitor biofilms regularly to avoid their formation. An alternative tactic could be the use of biocides with enough penetration power into the biocide. For this purpose, monochloramine cannot be recommended, although it has a reputation for biofilm penetration.

Acknowledgements

The model system was donated by Dizayn Teknik Plastic Pipes & Fittings Co. This study was supported by the Research Fund of İstanbul University (Project No: BYP-2251).

Corresponding author:
Esra İLHAN SUNGUR
Department of Biology,
Faculty of Science,
İstanbul University,
İstanbul - TURKEY
E-mail: esungur@istanbul.edu.tr

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